Studies on Cirrhosis of the Liver

I. Production and Treatment of Fatty Liver Cirrhosis of Albino Rats

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Recently there is a growing conviction that viral hepatitis plays an important role in the causation of cirrhosis of the liver. However, all hepatic cirrhosis is not ascribed to viral hepatitis.

The development of cirrhosis from fatty liver is strongly supported by American and European investigators from the experimental studies in choline-deficient rats and clinical observations. In this country Ueda¹⁾ reported 8 cases of fatty cirrhosis among 28 cirrhotic patients. The author experienced 2 clinical cases and 3 autopsied cases. Although Amano²⁾ maintains that fatty cirrhosis is rarely found in post-mortem material, it seems to be not a rare disease in Japan.

For the purpose of reexamining the problem of the production and treatment of fatty liver cirrhosis, fatty liver cirrhosis was produced in rats by feeding a choline-deficient diet, and then these rats received the treatment of high protein diet with added choline. An attempt was made to evaluate the effects of protein and choline not only by histological examination but also by determinations of liver collagen, liver fat, and serum protein.

EXPERIMENTAL

Methods

Fifty-two male albino rats with an average weight of 150 g. were used. They were fed with a choline-deficient diet for 8 months. Some of the rats were then killed and the remaining animals were fed with the therapeutic diet containing 30 per cent casein and 40 mg. of choline chloride and killed after 6 months. Histological examination and determinations of liver fat, liver collagen, and serum protein were all carried out on these animals. For normal controls, 14 male albino rats with an average weight of 130 g. were used. These rats were fed with a diet

similar to the therapeutic diet for 3 to 4 weeks, and were then sacrificed.

The composition of experimental diets employed is shown in Table I. Determination of liver fat was carried out by the method described

TABLE I
Composition of Diets

Constituent	Choline-deficient diet	Therapeutic diet	Diet for normal control rats
Casein	8%	30%	30%
Sucrose	48%	61%	61%
Lard	38%	—	—
Cod liver oil	2%	—	—
Soybean oil	—	5%	5%
Osborn-Mendel's Salt Mixture	4%	4%	4%
Choline chloride	—	40 mg/day	20 mg/day
Thiamin	50 μ g/day	50 μ g/day	50 μ g/day
Riboflavin	50 μ g/day	50 μ g/day	50 μ g/day
Pyridoxin	20 μ g/day	20 μ g/day	20 μ g/day
Calcium pantothenate	100 μ g/day	100 μ g/day	100 μ g/day
Nicotinamide	50 μ g/day	500 μ g/day	500 μ g/day
Percomorph liver oil	—	6 drops/week	6 drops/week
α -tocopherol	10 mg/week	5 mg/week	5 mg/week

by Kumakawa and Sudo, and that of liver collagen was done by the method of Lowry.³⁾ Serum protein was estimated by the method of paper electrophoresis and refractometry. Paper-electrophoretic analyses were made using phosphate buffer of pH 7.8.

Results

Body weight, food intake, and deaths of rats

Fig. 1 illustrates the curves of average body weight and food intake as well as deaths of rats. The body weight curve of the rats on the choline-deficient diet rised gradually and attained a weight of 180 g. after 10 weeks. Then there was no fluctuation of the body weight curve for 9 weeks. From the 20th week it declined slowly up to the initial weight after 8 months. When their diet was changed from the choline-deficient diet to the therapeutic diet, there was an abrupt acceleration of growth of the rats. After 18 weeks their body weight reached 333 g., and thereafter there was no increase of body weight.

The food intake of the choline-deficient rats remained unchanged for 10 weeks, and had a tendency to decrease from the 11th week. The

Fig. 1. Body weight, daily food intake and deaths of rats,
(Deaths: ● fibrosis, □ kidney injury, △ massive hepatic necrosis, ○ other)

average daily food intake was 8.3 g. The food intake of the rats receiving the therapeutic diet had a general trend to increase, the average intake being 14.1 g.

As is shown in Fig. 1, fibrosis of the liver was first recognized histologically in a rat which died after 18 weeks, and macroscopically in a rat which died after 25 weeks. The deaths of rats from fibrosis increased as they lost weight. Certain number of the rats on the choline-deficient diet developed alopecia from the 11th week, and after 8 months about half of them suffered from alopecia and the rats generally became ill. The change to the therapeutic diet brought prompt recovery, and alopecia disappeared in 2 to 5 weeks.

During feeding the choline-deficient diet, 10 mg. of α -tocopherol per week was given to prevent dietary massive necrosis of the liver. When α -tocopherol was decreased to 5 mg. per week for a short period, a rat developed massive necrosis and died.

Pathology

The liver of the rats fed with the choline-deficient diet was swollen and showed pale yellow color. These macroscopic findings of fatty liver were observed in almost all the rats which were fed with the choline-deficient diet for more than 4 months. The liver of about one-quarter of the rats showed finely granular surface and increased consistency, suggesting fibrosis of the liver macroscopically. (Fig. 2) In the treated rats macroscopic findings of fatty liver were not observed, but finely granular liver was still present. Marked nodular hyperplasia developed in a rat fed with the therapeutic diet for 6 months. (Fig. 3)

Histologically fatty metamorphosis was the most conspicuous finding

Fig. 2

Fig. 3

Fig. 2. Fatty liver cirrhosis of a rat fed with the choline-deficient diet for 6 months. Surface is finely granular, and consistency is increased.

Fig. 3. Liver of a rat fed with the therapeutic diet for 8 months, showing nodular hyperplasia of Lobus caudalis dexter. The liver is injected with India ink through the hepatic vein.

in the choline-deficient rats. Fatty metamorphosis began in centrolobular areas and finally fatty cysts occupied almost all parenchyma. Fatty metamorphosis appeared in all the rats fed with the choline-deficient diet for more than 4 months. In the livers of the rats which died after the relatively long period of starvation, only a few number of fatty cysts were found even when they were fed with the choline-deficient diet for more than 4 months.

Besides fatty metamorphosis, "ceroid" described by Lillie⁴⁾ appeared. This material occurred in round and oval globules, and was pale yellow with hematoxylin and eosin stain and brownish-orange when stained with sudan III. It was still stained with sudan III in paraffin sections. Ceroid was scattered when it first appeared. As ceroid became marked, it formed trabeculae which divided the lobules of the liver. This ceroid seemed to be phagocytized by Kupffer's cells and was present in sinusoids. The reticular fibers of the sinusoids which were filled with ceroid had the tendency to formation of collagenous fibers.

Ceroid appeared first in centrolobular and subcapsular areas and around hepatic veins as same as fatty metamorphosis did, and later periportal areas were involved. In some rats ceroid accumulation was so marked in the areas near the hilus of the liver that the parenchyma of those areas were totally replaced by ceroid and collagenous fibers. Although fibrosis generally developed with ceroid, in some instances only collagenous fibers were present without the appearance of ceroid. Generally the proliferation of connective tissue was not marked. (Fig. 4)

Fig. 4. Microphotograph of the liver of a rat fed with the choline-deficient diet for 8 months. Van Gieson's stain. $\times 64$.

Fibrosis began in non-portal areas as same as ceroid accumulation. Trabeculae of collagenous fibers and ceroid linked with adjacent central veins and surrounded the periportal triads. Later the periportal triads were also involved.

As fibrosis and ceroid accumulation progressed, trabeculae of connective fibers and ceroid divided hepatic lobules and formed pseudolobules. Thus the normal architecture of liver lobules was destroyed.

On the other hand regeneration of liver cells was noticed in some rats with fibrosis. Proliferation of bile ducts developed in the rats in which fibrosis and ceroid accumulation were advanced. In some instances atypical hyperplasia of bile ducts was present. Inflammatory cells were found only slightly.

The change of the choline-deficient diet to the therapeutic diet brought the rapid disappearance of liver fat and regeneration of liver cells. In some rats fibrosis was so delicate and without the presence of ceroid that the resorption of collagenous fibers and ceroid to a certain extent were conceivable. Nevertheless in other many rats fibrosis and ceroid accumulation were still marked. (Fig. 5) Generally no statistically significant changes were noticed in fibrosis and ceroid accumulation during the treatment, although the decrease of fatty metamorphosis was obvious. (Table II)

In the liver of a rat which died from massive hepatic necrosis, diffuse coagulation necrosis of liver cells and hemorrhage were both noticed.

In the spleen ceroid was phagocytized by reticular cells around white pulps, in white pulps, and in splenic cords. Fibrosis of white and red pulps developed in a treated rat.

The renal lesion of the rats with hemorrhagic degeneration of Griffith⁵⁾ was characterized by dark and enlarged kidneys, necrosis and degeneration of tubules, hemorrhages in capsules and cortex, and cast on the one hand,

Fig. 5. Microphotograph of the liver of a rat which received treatment for 6 months. Van Gieson's stain. $\times 68$.

TABLE II
Fatty Metamorphosis, Ceroid Accumulation and Fibrosis
of the Liver Estimated Histologically

Group	No. of rats	Fatty Metamor- phosis					Ceroid Accumu- lation					Fibrosis				
		0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Normal control rats	14	14	0	0	0	0	14	0	0	0	0	14	0	0	0	0
Rats died during the first 4 months of the choline-deficient diet	11	9	0	1	1	0	11	0	0	0	0	11	0	0	0	0
Rats died during the second 4 months of the choline-deficient diet	12	0	4	3	3	2	2	3	3	3	1	3	2	6	1	0
Rats fed with the choline-deficient diet for 8 month	12	0	1	5	4	2	3	3	5	1	0	3	2	6	1	0
Rats died during the treatment	8	5	3	0	0	0	3	0	2	3	0	3	0	3	0	2
Rats treated for 6 months	9	8	1	0	0	0	1	3	4	1	0	3	2	4	0	0

and by fibrosis of cortex on the other hand. The latter seemed to be the result of scarring of the former changes. Besides these changes there was ceroid deposition in glomerulus and calcification of vessels, but glomerulosclerosis was not observed.

In the lung ceroid and fat deposited in the septa. Parenchymatous myositis, cellular infiltration and fibrosis were all present in the heart of some rats. Atrophy of testis was found in the rats in which fibrosis was marked.

Liver fat

Among the rats died within the first 4 months which were fed with the choline-deficient diet, only a few rats showed the increase of liver fat, probably because of their small food intake. Their average daily food intake was 4.3 g.

Liver fat increased over normal range in all the rats which died during the second 4 months and those which were killed thereafter. The average fat content of the liver of the rats which died within the second 4 months of the choline-deficient diet was 23.0 per cent, and that of the rats killed after 8 months was 24.8 per cent. Thus the increase of liver fat in the rats fed with the choline-deficient diet for more than 4 months was clearly demonstrated. (Table III)

TABLE III
Liver Weight, Liver Fat and Liver Collagen

Group	No. of rats	Average final body weight, g.	Average weight of liver, g.	Liver weight as per cent of body weight	No. of rats	Liver fat as per cent of liver	No. of rats	Liver collagen as per cent of liver
Normal control rats	14	216.9	9.6	4.4	13	6.2	12	0.30
Rats died during the first 4 months of the choline-deficient diet	11	90.4	5.5	6.2	6	10.8	—	—
Rats died during the second 4 months of the choline-deficient diet	12	86.0	8.3	9.0	9	23.0	—	—
Rats fed with the choline-deficient diet for 8 months	12	137.4	9.7	7.5	12	24.8	11	0.33
Rats died during the treatment	8	160.0	9.3	6.0	7	6.4	—	—
Rats treated for 6 months	9	334.6	13.4	3.9	9	6.4	9	0.33

Dietary treatment was followed by the prompt and marked decrease of liver fat. The fat content was within normal range in all the rats which died during the treatment except two rats which died after 2 and 14 days and in all the rats which were killed after treatment except a rat which showed only mild increase of liver fat.

The relation of fat content to fibrosis or ceroid accumulation is interesting, as shown in Figs. 6 and 7. The differences of content of liver fat between the group of minimal fibrosis and that of moderate fibrosis, between the group of minimal ceroid accumulation and that of moderate ceroid accumulation, between the group of moderate ceroid accumulation and that of advanced ceroid accumulation were statistically significant. Thus the content of liver fat decreased as fibrosis or ceroid accumulation

Fig. 6. The relation between fibrosis of the liver and liver fat in the rats fed with the choline-deficient diet for more than 4 months.

Fig. 7. The relation between ceroid accumulation and liver fat in the rats fed with the choline-deficient diet for more than 4 months.

progressed.

The degree of fatty metamorphosis which was estimated histologically was proportional to the content of liver fat which was determined chemically.

Collagen content

Since liver collagen content was determined in a few rats of those which died during the feeding of the choline-deficient and therapeutic diets, comparisons were made between the normal control rats, the rats fed with the choline-deficient diet for 8 months, and the rats fed with the therapeutic diet for 6 months. Their average collagen contents were 0.30, 0.33, and 0.33 per cent respectively.

When tested by Fisher's "t" test, the differences of collagen content between the normal control rats and the rats which were fed with the choline-deficient diet for 8 months, between the rats which were fed with the choline-deficient diet for 8 months and the rats treated for 6 months were all statistically significant. (Table III)

Serum protein

The differences of total protein concentration between the three groups were statistically significant. (Table IV) Total protein concentration was found decreased in the rats which were on the choline-deficient diet, and increased over normal value in those which were on the therapeutic diet.

Albumin concentration was found decreased markedly in those which

TABLE IV
Serum Protein

Group	No. of rats	g/dl					No. of rats	%				A/G
		Total	Al.	α -Gl.	β -Gl.	γ -Gl.		Al.	α -Gl.	β -Gl.	γ -Gl.	
Normal control rats	14	6.64	2.89	0.74	1.36	1.65	14	43.6	11.1	20.5	24.8	0.77
Rats fed with the choline-deficient diet for 8 months	9	6.10	2.13	0.76	1.37	1.84	21	33.0	13.4	22.5	31.1	0.50
Rats treated for 3 months	—	—	—	—	—	—	11	33.5	11.7	22.8	32.0	0.51
Rats treated for 6 months	9	7.69	2.99	0.85	1.55	2.29	9	38.9	11.1	20.1	29.9	0.64

were on the choline-deficient diet, and returned to normal in those which were on the therapeutic diet.

There were no significant changes in α -globulin concentration.

β -Globulin concentration remained unchanged in those on the choline-deficient diet. The difference of β -globulin concentration between the treated rats and the control rats was statistically significant, whereas the difference between the treated rats and the choline-deficient rats was not significant.

γ -Globulin concentration was not found increased in those which were on the choline-deficient diet, and on the contrary was increased significantly in those which were on the therapeutic diet.

After 3 months of the treatment only percentage of the serum protein was estimated. Judging from the results, the changes of serum protein of the treated rats seemed to be more pronounced in the first 3 months than in the second 3 months.

Albumin-globulin ratio was found lowered in those which were on the choline-deficient diet, and remained unchanged in the first 3 months of the treatment. In the second 3 months of the treatment albumin-globulin ratio rised, but failed to return to normal.

In the rats which were fed with the choline-deficient diet for 8 months there were no statistically significant correlations between albumin and γ -globulin concentration, between albumin concentration and fibrosis or ceroid accumulation, and between γ -globulin concentration and fibrosis or ceroid accumulation.

DISCUSSION

The histological changes in this experiment were characterized by the fibrosis which began in centrolobular areas and ceroid accumulation.

The non-portal distribution of fibrosis in experimental cirrhosis were recognized by Ashburn and his associates⁶⁾ and confirmed by other investigators.^{7),8),9)} In this regard the difference of experimental cirrhosis from human cirrhosis needs to be discussed.

According to Popper and his associates,¹⁰⁾ fatty cirrhosis of man is portacentral, and necrosis and inflammation in periportal and intralobular areas and membrane formation in stress fissures caused by uneven expansion of hepatic territories were more important processes of development of fibrosis than the collapse of fatty cysts. In this experiment the former two processes were not observed. Since fibrosis began in the areas of the most pronounced fatty metamorphosis and fat content decreased as fibrosis progressed, a close relation seems to be present between fibrosis and fatty metamorphosis itself. Hence the process of development of fatty cirrhosis in rats appears to be different from that of man.

However, in the experiences of the present author in human liver cirrhosis there have been no findings which indicated, as Popper and his associates described, that periportal and intralobular necrosis and inflammation and membrane formation were the main processes of development of fibrosis. Further investigation is required on the problem concerning the processes of development of fibrosis in man.

Deposition of ceroid in large quantities in livers seems to be due to the use of cod liver oil and lard in the diet.⁷⁾ Since experimental fatty cirrhosis without ceroid can be produced on certain diets,^{7),11)} it is unlikely that ceroid plays a role in the causation of fibrosis in experimental cirrhosis, and that the presence of ceroid in experimental cirrhosis is an evidence against identity with human cirrhosis.

The coexistence of both fibrosis and ceroid in most cases suggests that the same cause as that of fibrosis, such as the rupture of fatty cysts described by Hartroft,¹²⁾ contributes to the formation of ceroid in certain conditions, for instance, when cod liver oil or lard is used.

The determination of liver fat content clearly shows the lipotropic activity of choline and high protein diet. Noteworthy is the relation of fat content to fibrosis and ceroid accumulation. Fat content decreased as fibrosis and ceroid accumulation progressed. This contributes, as well as the prompt decrease of fat after the treatment and starvation, to the rapid disappearance of the characteristics of fatty cirrhosis. Caution must be taken about this point in the investigation of etiology of liver cirrhosis.

In this experiment collagen content was expressed in per cent of wet liver weight as it was considered to be most adequate. To evaluate the changes of collagen content the influences of changes of liver fat and body weight on collagen content must be considered. Since the increase of liver fat decreases apparent collagen content, the collagen content of

the rats which were fed with the choline-deficient diet seems to be actually increased over the above-mentioned figures, and that of the treated rats seems to be actually decreased below the above-mentioned figures. However, the collagen content of the livers of the treated rats was increased over that of the normal control rats, whereas the fat content of the former was the same as that of the latter. Therefore, the changes of liver collagen similar to that of the choline-deficient rats seem to be not present in the treated rats.

According to Harkness and Harkness¹³⁾ liver collagen (mg.) in normal rats is proportional to liver weight (g.).^{1,16} When liver collagen (mg.)/liver weight (g.)^{1,16} is calculated, it is 2.08 in the normal control rats, 2.28 in the choline-deficient rats, and 2.20 in the treated rats. The degree of decrease in the treated rats is mild as compared with the degree of increase in the choline-deficient rats.

Thus, when the influences of liver fat and body weight on the collagen content are considered, the decrease of collagen content in the treated rats is not clearly demonstrated, whereas the increase of collagen content in the choline-deficient rats is apparent.

There have been no reports on the changes of serum protein in the experimental cirrhosis. When the results in this experiment are compared with those in clinical cases with liver cirrhosis^{1),14),15),16),17),18)} or fatty liver,¹⁰⁾ the former are generally accorded with the latter.

It may be based upon the fact that fibrosis is of mild degree and that, as reported by Ueda,¹⁾ the cirrhosis is a fatty cirrhosis, that the difference of γ -globulin concentration between the choline-deficient rats and the normal rats was not significant. The changes of serum protein on the therapeutic diet are in accord with those in clinical cases.^{1),15),19),20)}

In clinical cases when they are examined by serial biopsies fibrosis are reported to remain unchanged after dietary and lipotropic treatment,^{19),21),22),23),24),25),26)} whereas in experimental cirrhosis decrease of fibrosis was reported by some investigators.^{9),27),28),29),30),31),32)}

In this experiment the decrease of fibrosis was not demonstrated in agreement with the results in many clinical cases and the experiment of Lowry.³³⁾ The decrease of fibrosis was further confirmed by the determination of collagen content of livers. On the basis of the experimental findings it is suggested that the decrease of fibrosis after the dietary and lipotropic treatment can not be expected once fibrosis has progressed to a certain extent, though the functional and morphological improvement of liver cells many occur.

SUMMARY AND CONCLUSIONS

1. Albino rats were fed with a choline-deficient diet for 8 months.

Some of the rats were then killed and the remaining animals were fed with the therapeutic diet containing 30% of casein and 40 mg. of choline chloride for 6 months. Microscopic examination and quantitative determination of liver fat, liver collagen and serum protein were carried out on these animals.

2. The liver of the rats which were fed with the choline-deficient diet was finely granular, and microscopic examination showed fatty metamorphosis, ceroid accumulation, and fibrosis of the liver which began in non-portal areas. The dietary treatment was followed by the rapid disappearance of fat, but there were no recognizable effects on fibrosis and ceroid accumulation.

3. Liver fat increased remarkably on the choline-deficient diet, and returned to nearly normal level on the therapeutic diet. In the rats which were fed with the choline-deficient diet for more than 4 months liver fat decreased as fibrosis or ceroid accumulation progressed.

4. Liver collagen increased on the choline-deficient diet, but the decrease on the therapeutic diet was not clearly demonstrated.

5. On the choline-deficient diet serum albumin decreased markedly, but the increase of γ -globulin was not statistically significant. After the dietary treatment albumin returned to normal and γ -globulin increased significantly. Albumin-globulin ratio was lowered on the choline-deficient diet. On the therapeutic diet albumin-globulin ratio rised significantly, but failed to return to normal.

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