EFFECTS OF NUCLEIC ACID PRECURSORS ON LIVER INJURIES

I. TREATMENT OF LIVER INJURY INDUCED BY LOW PROTEIN, AMINO ACID IMBALANCED DIET WITH AICA AND AICA OROTATE IN MASSIVE DOSES¹

SHUJI HASHIMOTO²

*Second Department of Internal Medicine, Kagoshima
University School of Medicine, Kagoshima*

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It has been known that nucleic acids play an important role in protein biosynthesis, since studies were made by Brachet (1), Caspersson (2), and others. In recent years, Zamecnik *et al.*, (3, 4, 5) have clarified the significance of the amino acid-activating enzyme-soluble RNA-ribosome system in protein biosynthesis, and Jacob and Monod (6) discovered the messenger RNA. It has also been elucidated that ATP, GTP, CTP, UTP, *etc.* act as nucleotide coenzymes and participate in a variety of metabolic reactions.

It is therefore presumed that RNA plays a significant role in the regeneration and recovery of damaged tissues. In acute hepatic injuries, for instance, more C¹⁴-orotate is incorporated into the liver nucleotide than normal state, no matter how the liver protein and RNA levels decrease. This may indicate that excessive RNA is required as regeneration proceeds and tissue nucleic acid metabolism is accelerated.

In healthy animals, nucleic acids are considered to be synthesized *de novo* in the body, but pyrimidine or purine bases, when administered to healthy animals, are not utilized.

However, since orotic acid has been reported to promote healing of liver injuries and growth of immature animals, it can be presumed that pyrimidine and purine bases or their precursors are utilized in cases of increased nucleic acid consumption.

The author also has recognized, both experimentally and clinically, the effectiveness of uridine or cytidine in the treatment of liver injuries (7, 8).

Standerfer *et al.* (9) reported on the development of fatty livers in animals maintained on a diet containing 1 % orotic acid, and Handschumacher *et al.* (10) postulated such symptoms to be induced by an unbalance of nucleic acid metabolism, since they can be prevented by a diet supplemented with adenine.

¹ Following abbreviations are used : AICA, 4-amino-5-imidazolecarboxamide ; OCT, ornithine carbamyl transferase

² 橋本修治. Present address : Department of Physiology, School of Medicine, Western Reserve University, Cleveland 6, Ohio, U.S.A.

The author (11) observed experimentally that growth inhibition, disturbance of nucleic acid and riboflavin metabolisms occurred in normal rats receiving uracil in massive doses. The incidence of fatty livers due to orotic acid feeding has been proven to be inhibited by concomitant treatment with AICA.

As mentioned above, an overdose of pyrimidine may cause relative deficiency of purine bases, interferes with the biosynthesis of nucleic acids, inhibits various metabolic reactions, in which purine nucleotide coenzymes participate, and as a result, brings about fatty liver and growth inhibition.

This paper presents studies on the influence of AICA and AICA orotate on rats with liver injuries induced by feeding on low-protein and amino acid-imbalanced diet.

EXPERIMENTAL

Rats of the Wistar strain, each weighing about 200 g, were maintained on a low protein and amino acid imbalanced diet (Table 1) for a period of 11 weeks to induce fatty livers. The animals were then divided into 3 groups. Group I received a 20 % casein diet (Table 1) alone; Group II, the same diet plus AICA

TABLE 1
Compositions of Diets

	Low-protein, amino acid-imbalanced diet	20 per cent casein diet
	<i>g</i>	<i>g</i>
Casein	6.0	20.0
L-Cystine	0.2	—
Cod liver oil	2.0	2.0
Colza oil	2.0	2.0
Vitamin mixture ^a	1.0	1.0
Choline chloride	0.1	0.1
Salt mixture ^b	4.0	4.0
Sucrose	15.0	15.0
Potato starch	69.7	55.9
Total	100	100
Mixed with water	37.5	37.5

Administered daily 32 g for each rat.

^a Vitamin Mixture		^b Salt Mixture (14)	
	<i>mg</i>		<i>mg</i>
Thiamine-HCl	1.00	CaCO ₃	29.29
Riboflavin phosphate	1.33	Ca(H ₂ PO ₄)·H ₂ O	0.63
Pyridoxine-HCl	1.00	KH ₂ PO ₄	34.31
Niacin	6.67	NaCl	25.06
Folic acid	0.17	MgSO ₄	4.87
Na pantothenate	1.67	Reduced Fe	0.139
Thioctic acid	1.33	CuSO ₄	0.100
Cyanocobalamin	0.67 (μg)	MnSO ₄ ·4H ₂ O	0.160
Ascorbic acid	37.50	ZnCl ₂	0.02
Menadione	0.20	KI	0.0005
PABA	10.00	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.0025
Biotin	15.00 (μg)	Total	94.582
Inositol	15.00		
Dextrin	q.s.		
Total	1,000.00		

(1 %); and Group III, the same diet plus AICA orotate (2 %) for further 4 weeks.

Some of these animals in each group were killed at the 3rd, 5th, 7th, 9th, 11th and 15th weeks of maintenance, and the following studies were carried out. Dosages of AICA and AICA orotate used in this experiment roughly corresponded to 1600 and 3200 mg daily per kg body weight, respectively.

RESULTS

1. Changes of Liver Lipids

Low protein diet feeding increased the liver phospholipid level slightly and the neutral lipid and cholesterol levels markedly (Fig. 1).

There was no notable fluctuation in the phospholipid level after the 3rd week, but the neutral lipid and cholesterol ester levels reached a maximum at the 3rd and the 7th weeks, respectively, and then began to decrease. This may indicate the development of fatty necrosis.

Upon institution of the 20 % casein diet, increase in the neutral lipid and cholesterol ester levels continued in Group I (control), but did not in Groups II and III.

In general, iodine numbers of fatty acid contained in the liver gradually decreased in animals fed with a low protein diet. This decrease was especially marked in the neutral lipid and cholesterol ester fractions. After feeding with the 20 % casein diet in Group I, the iodine numbers in the phospholipid fraction

FIG. 1 Changes of Liver Lipids
—, control; ---, AICA-treated group;
· · ·, AICA orotate-treated group.

FIG. 2 Changes of Liver Hexosamine and Collagen
—, control; ---, AICA-treated group; · · ·, AICA orotate-treated group

tended toward recovery, whereas those in the neutral lipid fraction still continued to decrease.

In Groups II and III, iodine numbers in the phospholipid fraction increased less than in Group I, but those in the neutral lipid fraction increased more than in Group I (Table 2).

TABLE 2
Changes of Iodine Numbers of Fatty Acids in the Liver

	Low protein diet			20 percent casein diet ^a		
	Normal	3 weeks after	11 weeks after	Control	AICA-treated group	AICA orotate-treated group
Total lipid	146.2	88.9	62.7	72.6	68.4	76.5
Phospholipid	117.1	64.7	57.6	77.1	72.0	72.0
Neutral lipid + cholesterol	168.8	76.8	70.8	66.8	73.1	80.5

^a 15 weeks after receiving 20 per cent casein diet for the last 4 weeks.

2. Liver Fibrogenesis

Feeding with the low protein diet gradually increased the liver hexosamine and collagen levels, which suggested the development of liver fibrogenesis (Fig. 2).

After feeding with the 20 % casein diet, both the liver hexosamine and collagen levels tended to show a decrease in Group I, while in Group II the liver hexosamine level still showed an increase and the collagen level less decreased than in Group I. In Group III, the liver hexosamine level still continued increasing, but the liver collagen level decreased to nearly the same extent as that in Group I.

3. Other Changes

The liver protein level continued to increase for 3–5 weeks and thereafter decreased, but tended to show a slight increase again after the 9th week. There were no marked differences among the three groups with respect to liver protein levels following feeding with the 20 % casein diet (Fig. 3).

The liver DNA level showed scarcely any fluctuation by feeding with the low protein diet, but was found to decrease in Groups I and III after feeding with the 20 % casein diet.

The liver RNA level increased at the 3rd week, then decreased, and again increased after the 7th week. After the low protein diet was switched over to the 20 % casein diet, the RNA level decreased again in Group I, but not in Group III (Fig. 4).

The serum OCT activity rose from the 3rd to 11th weeks, which phenomenon may be indicative of liver cell necrosis, especially degeneration of mitochondria. An apparent decrease in the OCT activity occurred in all groups after feeding with the 20 % casein diet. In Groups II and III, the decrease was more marked than in Group I (Fig. 5).

FIG. 3 *Changes of Liver Protein*

—, control; ---, AICA-treated group;
 - - -, AICA orotate-treated group

FIG. 4 *Changes of Liver RNA and DNA*

—, control; ---, AICA-treated group;
 - - -, AICA orotate-treated group

FIG. 5 *Changes of Serum OCT Activity*

—, control; ---, AICA-treated group, - - -, AICA orotate-treated group

4. Histologic Findings of Liver

Marked vacuolar degeneration of liver cell (Hematoxylin-eosin stain) and slight proliferation of collagen fibers (Azan-Mallory stain) were noted at the 7th week after feeding with the low protein diet. In all groups, vacuolar degeneration reduced soon after feeding with the 20 % casein diet. Reduction of liver cell fibrogenesis also was apparent, but the effect of AICA and AICA orotate was unclear.

5. Electron-Microscopic Findings of Liver Cells (Figs 6—10)

At the 7th week of feeding with the low protein diet, numerous lipid drops of various sizes were found in the cytoplasm, and glycogen granules were scarcely seen. Palade granules decreased and were distorted in arrangement. In certain

FIG. 6 7th Week of Feeding with the Low Protein, Amino Acid Imbalanced Diet (18,400 $\times$)

Numerous lipid drops of varying sizes in the cytoplasm; absence of glycogen granules; Palade granules decreased and distorted in arrangement; mitochondria showing high electron-density.

N, nucleus, m, mitochondria, c.m, cell membrane, b.c, biliary canaliculus, L, lipid drop, g, glycogen granule, e.r, endoplasmic reticulum

FIG. 7 11th Week (15,600 $\times$)

Decrease of lipid drops in the cytoplasm; nearly normal number of mitochondria and glycogen granules; Palade granules unchanged.

FIG. 8 4th Week after Instituting 20 per cent Casein Diet Following 11 Weeks on Low Protein, Amino Acid Imbalanced Diet (18,100 $\times$)

Increased vacuoles in cytoplasm near the central zone of lobules; widened lumen in rough-surfaced endoplasmic reticulum.

FIG. 9 4th Week after Instituting 20 per cent Casein + 1 per cent AICA Diet Following 11 Weeks on Low Protein, Amino Acid Imbalanced Diet (20,800 $\times$)

Increase in number of rough-surfaced endoplasmic reticulum and Palade granules greater than in ordinary liver cells; rate of glycogen granules to cytoplasm lower than the control.

FIG. 10 4th Week after Instituting 20 per cent Casein + 2 per cent AICA Orotate Diet Following 11 Weeks on Low Protein, Amino Acid Imbalanced Diet (15,500 $\times$)

Increase of Palade granules similar to AICA-treated group; rate of glycogen granules to cytoplasm lower than the control.

cells, a substance showing the same electron-density as lipid was found in the Golgi area. In those cells rich in lipid drops, the mitochondria showed a higher electron-density than normal and the cells were irregular in size. At the 11th week, lipid drops in the cytoplasm decreased and a nearly normal number of glycogen granules reappeared. Electron-density in the mitochondria were also found to be nearly the same as normal, while Palade granules remained decreased and Golgi area and endoplasmic reticulum were found more developed than normal.

At the 15th week, cells in the peripheral zone of the liver lobules of Group I gave nearly normal findings except for an increase in lipid granules and in the cells in the central zone of lobules, vacuoles in the cytoplasm increased and the lumen of rough-surfaced endoplasmic reticulum widened. Lipid granules were less in number in Group II than in Group I. Increase in the number of Palade granules was remarkable in both Groups II and III, seemingly larger than in ordinary liver cells.

DISCUSSION

It is well known that animals maintained on a low protein diet develop fatty livers which gradually lead to cirrhosis of the liver. In humans also, protein deficiency may constitute an important factor in the causation of liver cirrhosis.

In the present study, the author gave a low protein and amino acid imbalanced diet to rats and recognized a rapid increase of liver neutral lipid and cholesterol ester and a gradual increase of liver hexosamine and collagen levels. Both

neutral lipid and cholesterol ester levels decreased after reaching these high levels. This phenomenon may indicate the development of fatty necrosis. In each lipid fraction a gradual decrease in iodine number of fatty acids was noted, *i.e.*, a decrease in unsaturated fatty acids.

The fatty degeneration of the liver induced by low protein, amino acid imbalanced diet may be ascribable to the lowered biosynthesis of enzyme-proteins which are essential to various metabolic processes. In animals fed with low protein, amino acid imbalanced diet, the liver protein level showed a marked decrease particularly in the mitochondria and, parallel to this, various abnormalities occurred such as reduction of butyrate oxidation, disturbance of phospholipid transport, inactivation of the TCA cycle, acceleration of acid polysaccharide metabolism, *etc.* (12).

For the *de novo* synthesis of pyrimidine and purine bases, aspartic acid, glutamic acid, glycine, *etc.* are required. In protein-deficient states, therefore, the production of pyrimidine and purine bases may also decrease. Furthermore, the phosphorylation of pyrimidine and purine bases or of their precursors via phosphoribosyl pyrophosphate may be interrupted by a decrease in the production of ATP due to the disturbed TCA cycle.

In fact, the liver RNA level showed a temporary increase at the initial stage of feeding with low protein, amino acid imbalanced diet and later a decrease, which resulted in lowered P^{32} -incorporation into liver nucleic acid (12).

Such fatty degeneration and fibrosis induced by low protein, amino acid imbalanced diet might have been corrected if proteins had been replenished in sufficient amounts at an earlier stage. According to Patek *et al* (13), diffuse fatty cirrhosis of liver induced by a 4 % casein diet given for 6 months, was restored to almost normal after feeding with a 25 % protein diet for further 4 weeks.

In this experiment, the fatty liver necrosis induced by low protein, amino acid imbalanced diet was corrected after 4 weeks of feeding with a 20 % casein diet, during which processes the liver neutral lipid and cholesterol ester levels increased temporarily in the control. However, such increases in the liver neutral lipid and cholesterol ester levels were inhibited by AICA or AICA orotate added to the 20 % casein diet (Groups II and III). Improvement of iodine number of fatty acids in liver lipids was promoted by AICA orotate, being especially remarkable in the fractions of neutral lipid and cholesterol ester.

This may indicate that AICA orotate has the ability to correct liver fatty necrosis induced by low protein diet, without increasing neutral lipid or cholesterol ester levels.

An increase in levels of liver neutral lipid and cholesterol ester is by no means desirable, although this occurs in the process of recovery. Therefore, such action of AICA orotate may be of therapeutic significance.

The animals, even after receiving a 20 % casein diet, showed no marked change in liver protein level, while they showed a decrease in liver RNA and DNA levels. This decrease may be transient and therefore might be normalized by continued feeding with a 20 % casein diet. No such decrease in liver RNA level was encountered in the AICA orotate supplemented group.

In all groups, electron-microscopic studies revealed recovery of the ribonucleo-protein granules (Palade granules) which participate intact in the process of protein biosynthesis. This recovery was especially marked in the AICA or AICA orotate supplemented group. Both AICA and AICA orotate seem to possess the ability to promote metabolism of nucleic acids (especially RNA) in the recovery process of liver injuries induced by a low protein diet. The *de novo* synthesis of liver nucleic acids was interrupted by feeding with a low protein diet, and was accelerated again by switching this diet over to a 20 % casein diet. AICA or orotic acid, administered on this occasion, might be utilized in the biosynthesis of nucleic acids.

In the case of liver injuries, both AICA and AICA orotate demonstrate the ability to promote liver nucleic acid metabolism, which effect, however, was below expectations. The reason for this may be due to the excessive dosage of AICA and AICA orotate used in this experiment. AICA, even in massive doses, is said to cause no such fatty livers as does orotic acid, but the development of a metabolic unbalance of nucleic acid may be unavoidable.

A rise in the serum OCT activity occurred in animals on low protein diet. This may indicate that liver cell necrosis caused a leakage of cellular enzymes into the serum. OCT activity in the AICA and AICA orotate supplemented groups decreased more rapidly than in the group receiving a 20 % casein diet alone, which may be ascribable to the ability of both agents to promote healing of liver cell necrosis. Liver collagen levels decreased to nearly the same degree in both the control group and AICA orotate supplemented group, but this decrease was less in the AICA supplemented group than in the above two groups. AICA had therefore no beneficial effect on fibrosis, but rather showed a tendency to inhibit its recovery. This also may have resulted from the exceedingly large dosage used in this experiment.

SUMMARY

AICA and AICA orotate were given in massive doses together with 20 % casein diet to rats with liver injuries induced by a low protein, amino acid imbalanced diet, and the effects of both agents appraised.

AICA orotate was apparently of value in recovering iodine numbers of fatty acids in liver lipids, increasing Palade granules and inhibiting fibrosis. Given in optimum doses, both agents may exhibit further effects.

AICA was effective in repairing cell necrosis but had rather an inhibitive effect on the recovery of fibrosis.

It is advisable not to use AICA alone to prevent metabolic unbalance of nucleic acids.

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