SCI. REP. KYOTO PREF. UNIV. (NAT. SCI. & LIV. SC) NO. 40, Ser. B p. 7 ~12 (NOV. 1989)

Blood Properties and Liver Cell Components in Process of Rat Hepatic Cirrhosis Produced by Low Protein Diets.

YOSHIHO KATAYAMA, KAYO TANAKA*, CHIZUKO SHIMOSHIMA**

and MARI SAIMEI***

Abstract

We conducted to estimate hematological and metabolic changes in the productive process of hepatic cirrhosis. Male Charles-River Crj-CD rats were fed 5 % gluten diet, 5 % casein diet, 5 % wheat-pattern amino acid mixture diet and 20% casein diet as control *ad libitum* for 29 weeks. After autopsy, hematological and biochemical determinations were performed.

Body weight gains of rats on experimental low protein diets were significantly lower than that of the control, showing 82g for LC-group, 12g for W-group and −6g for G-group as compared with 420g of the control group at the 20th experimental week. At the 29th experimental week, rats in W-and G-groups showed significant decreases in RBC counts, hemoglobin contents, hematocrits and serum protein. On the same week, RNA, DNA contents and RNA/DNA ratios in rat liver tissues of W-group and G-group did not differ from those of the control group. For LC-group, however, collagen contents in the liver had a tendency to slightly increase. These biochemical evidences were co-ordinate to histological findings.

(Received August 15, 1989)

Introduction

From nutritional point of view, we determined changes of liver tissues in rats fed low protein or low amino acid mixture diets for 3 to 4 weeks. It was presented previously that these rats on low protein or low amino acid mixture diets accumulated excess of fat within their livers (1 ~ 7). Furthermore, possible mechanism of producing fatty liver was suggested in our preceeding paper (5). Recently, we reported that several rats fed low wheat gluten and casein diets for long period produced hepatic cirrhosis (8). There were some differences in producing time of cirrhosis between varieties of foods and consequently in response of rats to foods.

Then we conducted to estimate hematological and metabolic changes in the process of cirrhosis production as a step of elucidation for producing mechanisms of hepatic cirrhosis.

Laboratory of Nutritional-Physiology and Biochemistry, Faculty of Living Science, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan.

* Faculty of the Sciences of the Living, Osaka City University, Osaka 583.

** Food and Nutrition, Mukogawa Women's Univ. Nishinomiya 663, Hyogo.

***Nagasaki Prefectural Women's Junior College, Narutaki, Nagasaki 850.

Materials and methods

Diets and animals : Four kinds of diets were used in this experiment. As shown in Table 1., the diets were 5 % wheat gluten (G-group) , 5 % casein (LC-group) diets and 20% casein diet for the control group. Wheat gluten and casein were 5 % level as dietary protein level except for the control diet. Additionally an another diet (5 % level) was consisted of wheat-pattern amino acid mixture in which amino acids composition was the similar to a protein of wheat gluten. The other ingredients were the similar in three diets except for the control diet and as follows : corn starch (Amicol C, purchased from Nichiden Chemical Co. Inc., Osaka) , 83.8% ; corn oil purchased from Ajinomoto Co. Inc., (Tokyo), 5 % ; vitamin and salt mixtures purchased from Oriental Yeast Co. Ltd. (Tokyo), 1 % and 5 %, respectively. The compositions of these mixtures were the same as those of Harper pattern (9). The choline chloride purchased from Sigma Chemical Co. (St. Louis) was 0.2%. In the case of the control diet, corn starch contents was 68.8%.

Table 1. Composition of Diets

	LOW PROTEIN DIET			CONTROL DIET
	W-DIET	G-DIET	LC-DIET	
PROTEIN	5 %WHEAT-PATTERN AMINO ACID MIXTURE	5 % GLUTEN	5 % CASEIN	20 % CASEIN
CORN OIL	5 %			5 %
SALT MIXTURE	5 %			5 %
VITAMIN MIXTURE	1 %			1 %
CHOLINE CHLORIDE	0.2 %			0.2 %
CORN STARCH	83.8 %			68.8 %
TOTAL	100.0 %			100.0 %

THESE DIETS CONTAINED 15,000 IU OF RETINOL AND 37.5 UG OF ERGOCALCIFEROL PER KG OF EACH DIET, RESPECTIVELY.
THE COMPOSITION(%) OF SALT MIXTURE: $CaCO_3$, 29.29; $CaHPO_4\ 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCL, 25.06; $MgSO_4\ 7H_2O$, 9.98; $Fe(C_6H_5O_7)\ 6H_2O$, 0.623; $CuSO_4\ 5H_2O$, 0.156; $MnSO_4\ H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005 ; $(NH_4)_6Mo_7O_{24}\ 4H_2O$, 0.0025.
THE COMPOSITION(%) OF VITAMIN MIXTURE: THIAMINE HCL, 0.059; RIBOFLAVIN, 0.059; NICOTINIC ACID, 0.294; CALCIUM PANTOTHENATE, 0.235; PYRIDOXINE HCL, 0.029; MENADIONE, 0.006; BIOTIN, 0.001; TETRAHYDROFOLIC ACID, 0.002; CYANOCOBALAMIN, 0.0002; MYOINOSITOL, 1.176; ASCORBIC ACID, 0.588; LACTOSE, 97.551.

Autopsy : Rats were starved for 14 hours prior to sacrifice. Autopsy was performed on the 4th and 29th experimental week under light anesthesia with sodium 5- ethyl- 5- (1-methylbutyl) - 2-thiobarbiturate (Ravonal, Tanabe Seiyaku Co. Ltd., Osaka). Blood was drawn from abdominal aorta of these rats. The blood obtained from each animal was allowed to clot in ice-box and serum was separated by centrifugation at 3000 r.p.m. for 20 min. at 4 ℃. Serum was stored in a refrigerator at below 4 ℃ prior to the biochemical assay. The livers of the animals were removed from the carcasses immediately after bleeding. The blood was quickly washed out from their livers using a filter paper dipped in ice-cooled physiological saline solution and washing medium was whipped up. Subsequently, the liver tissues were stored in a freezer at −30℃ until biochemical assay.

Hematological and Biochemical determination : RBC and WBC were counted in samples diluted with Hayem's and Türk's solutions according to usual methods (10). The protein of serum and liver tissue was estimated with crystalline bovine serum albumin (Sigma Chemical Co. Inc., St. Louis) as standard according to the method of Lowry et al. (11). Triacylglycerol was determined by a modified method of Van Handel and Zilversmit (12) with triolein as a standard.

Determinations of RNA, DNA and collagen : RNA, DNA and protein fractions in the liver were

separated by modified method of Schumidt-Thanhauser-Schneider (13, 14) . Liver tissues from which lipids were extracted with chloroform : methanol (2 : 1) were pretreated with 2 . 1 N and 0.7N perchloric acid and acid soluble fraction was separated out by centrifugation at 3000 r.p.m. for 10 min. Three ml of 0.3N KOH was added into the precipitate and the precipitate added 0.3 N KOH was incubated at 37℃ for 1 hour. Subsequently, pH was adjusted to 7.0. Then supernatant separated by centrifugation at 3000 r.p.m. for 10 min. was used as RNA fraction. For DNA fraction, the precipitate in final stage of RNA separation described above was treated with 4 ml of 0.5N perchloric acid and was boiled at 100℃ for 30 min. After centrifugation, consequent supernatant was obtained as DNA fraction. Assay of RNA and DNA was performed by Fleck and Munro's method (15) and Dishe's method (16), respectively. On the collagen determination, the final precipitate in DNA preparation stage was hydrolyzed with 40ml of 6 N HCl by autoclave at 124℃ (18 pounds) for 18 hours. The hydrolyzed mixture was neutralized to pH 7.0 and was diluted with distilled water into 100ml. This diluted solution was used for measurement of hydroxyproline by Hutterer and Singer's method (17). The collagen contents in the liver were calculated from the amounts of hydroxyproline.

Results

The body weights of rats fed the experimental low protein diets decreased and remained below the initial body weight during the 4th to 29th experimental period except for the control showing relatively short period in which LC-group rats were below the initial body weight, medium period in which W-group rats were below the initial body weight and longer period in which G-group rats were below the initial body weight, respectively. At the 4th experimental week, fat contents in these rat livers were in the order of W-group >> G-group > LC-group ≧ control group (Table 2). At the same experimental period, however, protein and triacylglycerol in the serum decreased significantly in contrast with the control group (Table 3). The order of triacylglycerol contents in the sera of these rats was W-group=G-group < LC-group < control group. This order of triacylglycerol contents in these rat sera was inversely related to the order of intensity of fat accumulation in the livers. Thereafter, body weight of each rat increased gradually. At the 20th experimental week, mean body weight gains were 82g for LC-group, 12g for W-group and −6g for G-group as compared with 420g of the control group.

Table 2. Lipids Composition of Rats fed Low Protein Diets for 4 weeks.

		LOW PROTEIN DIET GROUP			CONTROL GROUP
		W−GROUP	G−GROUP	LC−GROUP	
TG (mg/g)	LONG CHAIN	103±59*	36±25	9±2	11±3
	SHORT CHAIN	2± 1			1.5±0.5
CH (mg/g)	ESTER	3.0±1.6	5.3±1.6	3.8±0.4	1.8±0.6
	FREE	3.2±1.9			2.9±1.1

EACH VALUE REPRESENTS THE MEAN ± HALF RANGE OF THE CONFIDENCE INTERVAL(CONFIDENCE LIMIT) AT 95 % LEVEL.
* INDICATES STATISTICALLY SIGNIFICANT DIFFERENCE FROM THE CONTROL RATS AT $P<0.05$.

Table 3. Blood Properties of Rats fed Low Protein Diets for 4 weeks.

	LOW PROTEIN DIET GROUP			CONTROL GROUP
	W−GROUP	G−GROUP	LC−GROUP	
PROTEIN (g/dl)	5.6±0.2*	5.5±0.3*	6.1±0.3	6.8±0.8
TRIACYLGLYCEROL (mg/dl)	38±10*	33± 6*	47±19	59±11
CHOLESTEROL (mg/dl)	85±15	114±15	114± 3	92± 8

EACH VALUE REPRESENTS THE MEAN ± HALF RANGE OF THE CONFIDENCE INTERVAL(CONFIDENCE LIMIT) AT 95 % LEVEL.
* INDICATES STATISTICALLY SIGNIFICANT DIFFERENCE FROM THE CONTROL RATS $P<0.05$.

In many cases of the experimental rats except for the control, anemia was observed in progress of feeding period of low protein diets. As shown in Table 4., at the 29th experimental week, for example, the experimental groups showed decreases in RBC counts, hemoglobin contents in the blood and hematocrits, especially showing significant difference in RBC counts, hematocrits and hemoglobins of W- and G-groups from the control group. For LC-group, there was no accurate significance. WBC counts of G-group decreased at the 29th experimental week, while LC-group had a tendency to increase slightly. Furthermore serum protein contents in W- and G-groups decreased apparently as compared with the control.

Table 4. Properties of Blood (29 weeks)

	LOW PROTEIN DIET GROUP			CONTROL GROUP
	W−GROUP	G−GROUP	LC−GROUP	
RBC ($\times 10^4$)	666±105*	509±120*	626	820±83
WBC ($\times 10^2$)	82±26	66± 8*	101	79± 3
HEMATOCRIT (%)	35.1±4.2*	27.9±5.6*	33.1	45.5±7.1
HEMOGLOBIN (g/dl)	14.2±3.4*	12.5±1.2*	12.7	17.4±2.8
SERUM PROTEIN (g/dl)	5.7±0.5*	5.5±0.8*	—	8.3±0.2

EACH VALUE REPRESENTS THE MEAN ± HALF RANGE OF THE CONFIDENCE INTERVAL(CONFIDENCE LIMIT) AT 95 % LEVEL.
* INDICATES STATISTICALLY SIGNIFICANT DIFFERENCE FROM THE CONTROL RATS $P < 0.05$.

Table 5. Compositions of Liver Tissues of Rat fed Low Protein Diet for 29 weeks.

	LOW PROTEIN DIET GROUP			CONTROL GROUP
	W−GROUP	G−GROUP	LC−GROUP	
RNA (mg/g liver)	5.8±2.4	6.0±3.5	1.8±6.9	5.7±11.9
DNA (mg/g liver)	2.6±0.3	2.3±0.7	1.9±1.4	2.0±0.3
RNA/DNA	2.2±0.8	2.7±1.7	1.0±3.9	2.3±2.7
PROTEIN (mg/g liver)	105±15	109±16	112±32	148±55
COLLAGEN (μg/g liver)	748±580	560±216	1464±2719	1082±1180

EACH VALUE REPRESENTS THE MEAN ± HALF RANGE OF CONFIDENCE INTERVAL(CONFIDENCE LIMIT) AT 95 % LEVEL.

In the 29th experimental week (Table 5.), there were no significant differences for RNA or DNA contents and RNA/DNA ratios in rat liver tissues of W-and G-groups from the control. However these parameters of LC-group were smaller than those of the other two experimental groups. The protein of the liver tissues slightly decreased in all of the experimental rats as compared with the control. While, the collagen contents in the liver tissues had a tendency to decrease in W- and G-groups, but to inversely increase for LC-group rats.

On histological findings, during the 18 to 26th experimental week, hemorrhage, degeneration and massive necrosis in the hepatic parenchym were observed in rat livers of LC-and W-groups. There was a wide area of destructive parenchym. In wide region of these cases, acinal structure had disappeared. In addition, microscopical views of LC-group showed islands of remnants of hepatocytes in the masses of destructive tissues. The periportal areas showed bile duct proliferation and hyperplasia of connective tissues and also were infiltrated with round cells. But at the same experimental period, the extensive fat accumulation was found in the liver of G-group alone. In process of progression of feeding time of low protein diets, a rat in W-group showed scar formation in subcapsular regions.

Discussion

The MCV, MCH and MCHC were calculated from results of blood properties described above. As shown in Table 6. , these three parameters did not differ against the control group. From these data, erythrocytes might be unchanged in morphological properties (cell volume, Hb contents etc) between feeding of four kinds of diets with various protein levels and various qualities of protein. However erythrocyte counts significantly decreased and anemia was found in these rats. From these facts, the decrease of hematopoietic activity in born marrow of the rats, and intensities of hemorrhage and hemolysis may be considered one of the causes of anemia. We must consider question as to why does hemorrhage occur in W- and LC-groups but not in the G-group. In the livers, the parameters of protein/DNA, RNA/protein and protein ratios to the control in the liver (%) were calculated and these parameters are indicated in Table 7. The ratios of protein/DNA were lower in three low protein diet groups in contrast with the control. These low levels may mean a decrease of protein content in a cell under deficient condition of protein nutrition. RNA/protein did not differ in W- and G-groups from the control except for lower level (below 50% decrease of the control) in LC-group. For LC-group, ratios of RNA/DNA and RNA/protein were lower as compared with the control. Waterlow and Burrin et al. described previously that estimates of ratios of RNA/DNA and RNA/protein suggested protein synthesis capasity of tissue (18, 19) . From this, protein synthetic capasity of liver in LC-group might be decreased. While for LC-group, collagen slightly increased but not in W- and G-groups. In histological findings, LC-group showed hyperplasia of connective tissues in the destructive parenchym in the liver with together bile duct proliferation. These biochemical and histological evidences were co-ordinate each other and co-incident with the finding in our previous paper (8).

Table 6. Comparison of MCV, MCH and MCHC.

	Low protein diets			Control group
	W-group	G-group	LC-group	
MCV (μ^3)	52.7	54.8	52.9	55.5
MCH (pg)	21.3	24.6	20.3	21.2
MCHC (%)	40.4	44.8	38.4	38.2

Table 7. Comparison of protein/DNA, RNA/Protein and protein ratios to the control.

	Low protein diets			Control group
	W-group	G-group	LC-group	
Protein/DNA	42.6	46.3	58.1	72.3
RNA/Protein	0.059	0.059	0.016	0.039
Protein ratios to the control	74	76	76	100

Acknowledgment

We would like to thank the Ajinomoto Co. Ltd. for providing all of the crystalline amino acids.

References

1) Katayama, Y. , Matsumoto, N. and Koishi, H. : *J. Jpn. Soc. Food and Nutr.* **23**, *561 (1970) (in Japanese)* .

2) Katayama, Y. , Kajiwara, N. , Fujinaka, M. , Nagata, Y. , Matsudaira, T. and Koishi, H. : *Reports of the Sciences of Living, Osaka City Univ.* **22**, 5 *(1974) (in Japanese)* .

3) Katayama, Y. , Fujinaka, M. Nagata, Y. , Shimoshima, C. and Saimei, M. : *Reports of the Sciences of Living, Osaka City Univ.* **32**, *15~30 (1984)* .

4) Katayama, Y. , Kodama, K. and Kubo, Y. : *J. Nutr. Sci. & Vitaminol.* **25**, *229~241 (1979)* .

5) Katayama, Y. and Saimei, M. : *J. Nutr. Sci. & Vitaminol.* **25**, *525~542 (1979)* .

6) Katayama, Y. , Chisaka, Y. , Saimei, M. and Shimoshima, C. : *J. Jpn. Soc. Food and Nutr.* **31**, *587~595 (1978) (in Japanese)* .

7) Katayama, Y. , Fukuda, F. and Shimoshima, C. : *Reports of the Sciences of Living, Osaka City Univ.* **29**, 15~24 (1981) (in *Japanese*) .

8) Katayama, Y. , Saimei, M. and Shimoshima, C. : *Sci. Reports Kyoto Pref. Univ. No. 39, B 21~28 (1988)* .

9) Harper, A. E. : *J. Nutr.* **68**, *405~418 (1959)* .

10) Kanai, I. : *Rinsho-Kensa Teiyo (Text book of Clinical Patholgy) pp* Ⅵ *3~27, Kanahara Press, Tokyo (1980)* .

11) Lowry, O. H. , Rosenbrough, N. J. , Farr, A. L. and Randall, R. J. : *J. Biol. Chem.* **193**, *265~275 (1951)* .

12) Van Handel, E. , and Zilversmit, D. B. : *J. Lab. Clin. Med.* **50**, *152~157 (1959)* .

13) Schumidt, G. and Thanhauser, S. J. : *J. Biol. Chem.* **161**, *83 (1945)* .

14) Schneider, W. C. : *J. Biol. Chem.* **161**, *293 (1945)* .

15) Fleck, and Munro, : *Biochim. Biophys. Acta*, **55**, *571~583 (1962)* .

16) Dishe, Z. : *Milcu. Chemie*, **8**, 4 (1930) .

17) Hutterer, and Singer, : *Analytical Chem.* **32**, *556~558 (1960)* .

18) Waterlow, J. C. , Garlick, P. J. and Millward, D. J. : *Protein turnover in mammalian tissues and in the whole body, pp 538~550, Amsterdam, North Holland (1978)* .

19) Burrin, D. G. , Britton, R. A. and Ferrell, C. L. : *J. Nutr* . **118**, *1547~1552 (1988)* .