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Changes of Blood Fluidity in the Early Hepatopathy in Rats

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Abstract. *The present study was designed to examine the influence of the early stage hepatopathy on blood fluidity by using a rat experimental system. F344 male rats, 4 weeks of age, were fed chow containing 3'-methyl-4-dimethylaminoazobenzene (DAB) at 0.06%. These rats were autopsied 8, 12, 16 and 20 weeks after starting DAB feeding. Blood samples were collected from the inferior vena cava under pentobarbital anesthesia and blood fluidity and platelet aggregation activity were examined by a Micro Channel Array Flow Analyzer and a platelet aggregometer, respectively. We also examined histological changes in the liver after staining with hematoxylin-eosin. Histological observation of the liver revealed early-stage hepathopathy when the organs were obtained from rats that fed DAB for more than 16 weeks. Although DAB-feeding of rats for 8 and 12 weeks barely affected blood fluidity, long-term intake (>16 weeks) caused decrease in fluidity. On the other hand, platelet aggregation activity was increased when rats were fed DAB for more than 16 weeks. The present results suggest that assaying for blood fluidity may be useful for the assessment of the degree of hepatopathy.*

The liver is a vital organ present in vertebrates. It has a wide variety of functions, such as detoxification, glycogen storage and hormone metabolism. The liver also plays essential roles in blood coagulation through the production of fibrinogen that is responsible for coagulation.

Fatty liver disease (FLD) is a reversible condition where large vacuoles of lipid (1), primarily triacylglycerol, are accumulated in liver cells *via* the process of steatosis. Despite having multiple causes, excessive alcohol consumption is generally believed to be the most important factor in the development of FLD (2). On the other hand, nonalcoholic FLD is frequently found in people with metabolic syndrome, such as obesity, combined hyper lipidemia and diabetes mellitus (3). Nonalcoholic FLD refers to a wide spectrum of liver disease, ranging from simple fatty liver, nonalcoholic steatohepatitis (NASH) and cirrhosis (4). In NASH, fat accumulation is associated with varying degrees of inflammation and fibrosis of the liver (5).

It is reported that there are changes in platelet function in liver disorders, including viral hepatitis and liver cancer. However, there is little evidence showing the relationship between slight mild liver disorders, such as NASH, and platelet functions including blood fluidity. There is much evidence that long-term administration of 3'-methyl-4-dimethylaminoazobenzene (DAB) to rats causes a wide spectrum of liver disease, ranging from simple fatty liver to hepatic tumors (6, 7). Therefore, we induced early hepatic disorder by DAB administration in rats and examined the influence of the hepatic disorder on the platelet aggregation and the blood fluidity.

Materials and Methods

Experimental animals. Specific pathogen-free 4-week-old 70g male F344 rats were purchased from CLEA Japan Co. (Tokyo, Japan). The animals were maintained at 25±2˚C, humidity at 55±5%, and a light and dark cycle of 12 hours in our animal facilities. This study was approved by the Ethics Committee of Showa University for Animal Experiments (ID: 00071).

Induction of hepatopathy in rats. The hepatopathy model rats were made by DAB (Sigma Chem. Co,. St Louis, MO, USA) oral feeding. DAB was well mixed with regular powder diet (CE-2; CLEA Japan Co.) for maintaining rats and mice at a concentration of 0.06% (8). A group of rats (n=7) were fed chow containing DAB for 8, 12, 16 and 20 weeks *ad libitum*. These rats were then maintained with normal regular powder diet (CE-2) for a further two weeks and were used as the hepatopathy model (Figure 1). All rats were sacrificed under ether anesthesia when 26-weeks old. Blood and liver were collected. Blood and livers were collected under pentobarbital anesthesia.

Blood sampling and anticoagulant. A 3.5 ml blood sample was obtained from the inferior vena cava of the experimental rats anesthetized by intraperitoneal injection of pentobarbital sodium (Somnopentyl®; Kyoritsu Seiyaku Co., Tokyo, Japan) with a 22-

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Key Words: Hepatopathy, lood fluidity, platelet aggregation.

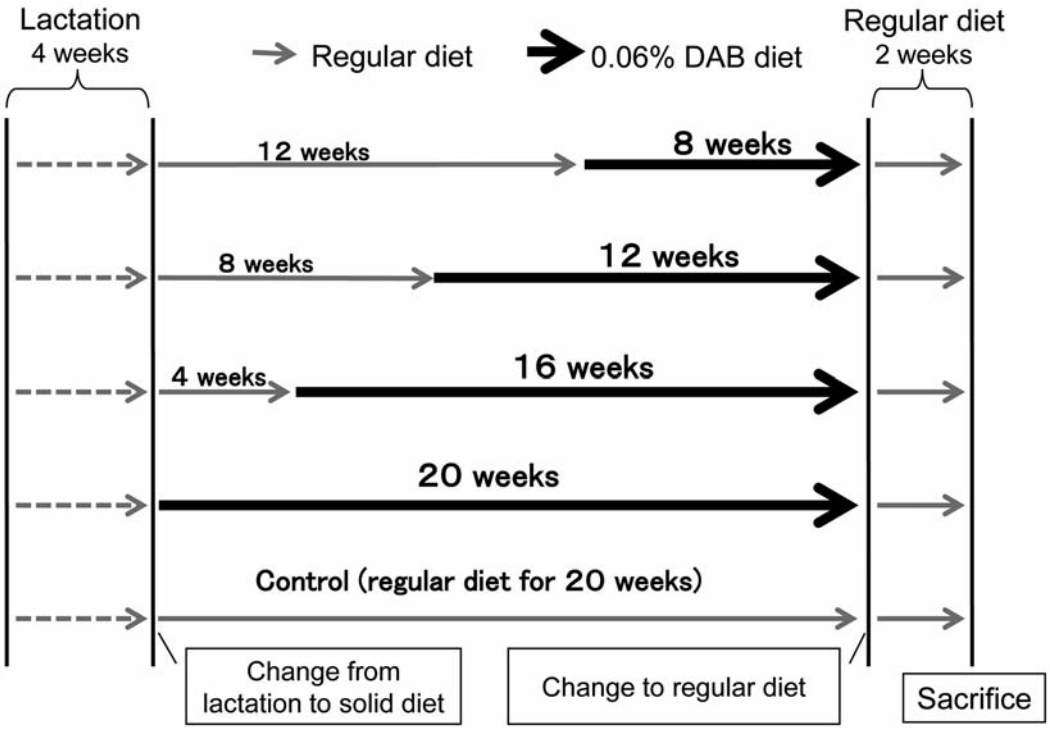

Figure 1. *Experimental procedure and schedules. The difference between groups was the period that they were fed the diet including 3'-methyl-4-dimethylaminoazobenzene (DAB). All animals were sacrificed at 26 weeks old of age (each group: n=7).*

gauge needle. To prevent coagulation of the blood, 45 units of sodium heparin were added to 1.0 ml of the blood sample, 2.4 mg of EDTA-2K to 0.5 ml, and 3.2% of sodium citrate to 2.0 ml.

Histological observation. Liver specimens were fixed in 4% paraformaldehyde-phosphate buffered saline (PBS) and embedded in paraffin. The tissue blocks were cut into 7-μm sections that were mounted on slides and stained with hematoxylin-eosin. The samples were observed under a microscope (BM-2; OLYMPUS Co. Ltd., Tokyo, Japan) for the verification of steatosis, hepatitis and heterogeneity.

Blood analysis. Red blood cells, white blood cells, platelets and hematocrit of the EDTA-2K-treated blood sample were counted by using an automatic blood cell analyzer for experimental animals (PCE-210; ERMA inc., Tokyo, Japan).

Analysis of liver enzymes. The plasma alkaline phosphatase (ALP) level was measured by using commercially available ALP assay kits (LabAssay™ ALP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's recommendations. The minimum detectable level of this kit was 0.06 mmol/l.

Measurement of blood fluidity. The blood fluidity was examined by using a KH-6 Micro Channel Array Flow Analyzer (MC-FAN; MC Laboratory Inc., Tokyo, Japan). Coagulation of blood was blocked by sodium heparin or EDTA-2K. We used 100 μl of the blood to measure the flow time to the silicon tip of the analyzer. It was assumed that the flow time in the analyzer imitated the capillary blood fluidity index (9). In an MC-FAN assay, the prolongation of flow time indicates a decrease of blood fluidity and a short flow time, an increase of blood fluidity (10).

Measurement of platelet aggregation activity. To prepare platelet-rich plasma (PRP) used for examination of platelet aggregation activity, the blood samples treated with sodium citrate were centrifuged at 400 ×*g*. After 5 minutes, PRP was obtained in a volume of 200 μl, and the remaining blood was further centrifuged at 2,300 ×*g* for 5 minutes. The supernatant (500 μl), thus, obtained was used as platelet-poor plasma (PPP). The number of platelet in PRP was adjusted to 3×10^6/μl with PPP and was used as samples for measurement of platelet aggregation activity.

Platelet aggregation was measured with a platelet coagulation measuring system, a platelet aggregometer (PA-20; Kowa Co. Ltd., Tokyo, Japan). Controlled PRP (270 μl) in a cuvette was pre-warmed to 37˚C. Adenosine diphosphate (ADP; Oriental Yeast Co. Ltd., Tokyo, Japan) was added at a final concentration of 10 μM, and the aggregation was measured. The aggregates measured were divided into three categories according to the size of aggregates: small (diameter=9-25 μm), medium (diameter=26-50 μm), and large (diameter=50-70 μm) (11, 12).

Statistical analysis. Continuous variables were presented as the mean±standard deviation of the mean (SD). The statistical significance between the control and the experimental groups was analyzed with analysis of variance (ANOVA) followed by the Fisher's protected least significant difference test. A *p*-value of less than 0.05 was considered statistically significant.

Table I. *Blood properties and body weight. The data are expressed as the mean±SD of 7 rats/group. *p<0.05 vs. control.*

Parameter	Control	DAB intake (weeks)			
		8	12	16	20
Platelet ($\times10^4$/μl)	50.75±8.76	50.84±6.43	51.38±8.54	48.07±5.38	45.37±4.02*
Leukocyte ($\times10^3$/μl)	3.61±1.17	3.68±0.91	3.01±0.09	3.06±0.77	3.30±0.59
Erythrocyte ($\times10^4$/μl)	740.48±61.30	753.84±31.76	738.65±56.23	761.91±19.84	680.75±32.85
Hematocrit (%)	38.82±3.46	40.68±2.16	43.76±3.90	42.48±1.28	39.20±2.01
Body weight (g)	293.75±23.19	266.76±14.61	266.21±19.21	268.03±13.14	277.01±16.43

Results

Histological observation of liver obtained from DAB-intake rats. The first experiments were carried out to examine whether oral feeding could induce hepatopathy in rats. To do this, rats were orally fed 0.06% DAB well mixed with chow. These rats were killed 8, 12, 16 and 20 weeks after starting DAB feeding. Although short-term (8 and 12 weeks) feeding of DAB did not cause any changes in liver (data not shown), long-term (>16 weeks) feeding caused remarkable lipid deposition in the liver (Figure 2). In order to obtain histological evidence for hepatopathy, liver sections were prepared and stained with hematoxylin-eosin; a typical image of liver of one out of seven rats is shown in Figure 3. The structure of the hepatic sinusoids and parenchyma cells were normal in the sections from untreated control rats. In contrast, the hepatic parenchyma cell and the nuclear size were altered in rats fed DAB for more than 16 weeks. Fatty metamorphosis and/or inflammatory cell infiltration were present in the liver of rats fed DAB for 20 weeks.

ALP levels in DAB-fed rats. The second experiments were designed to examine the influence of DAB feeding on the changes in plasma ALP levels. Plasma samples were obtained from rats fed DAB for 8, 12, 16 and 20 weeks, and the ALP levels were examined. As shown in Figure 4, ALP levels in plasma from experimental rats increased significantly when rats were fed DAB for more than 16 weeks as compared to that from controls.

The influence of DAB feeding on blood fluidity. The experiments were designed to examine the influence of DAB feeding on blood fluidity, and blood flow time was measured with an MC-FAN system. Firstly, blood samples were treated with heparin (Figure 5A). The blood flow time of the blood from control rats was 57.45±6.27 seconds; from the 16-week DAB intake group, 67.68±11.61 seconds; and from the 20-week DAB intake group, 57.69±4.40 seconds. Next, blood samples were treated with EDTA-2K (Figure 5B). No significant difference was observed between the control group and other groups.

The influence of DAB feeding on platelet aggregation. The experiments were designed to examine the influence of hepatopathy on platelet aggregation. The light transmission (Trans%) of PRP prepared from experimental rats showed an increase when compared with the controls (Figure 6A). The light scattering method revealed that the proportion of large-sized platelet aggregates of the DAB intake groups was significantly increased as compared with the control. In addition, the proportion of medium- and small-sized aggregates in the DAB intake groups decreased significantly (Figure 6B).

Basic blood characteristics. In blood fluidity experiments, it is important to consider factors which influence blood fluidity: the number of erythrocytes, leukocytes and platelets and the hematocrit. In rats fed DAB for 20 weeks, the numbers of platelets and erythrocytes significantly decreased compared to those of the control group (Table I).

Discussion

Nonalcoholic FLD is the most common form of early chronic liver disease which is associated with the incidence of insulin resistance and metabolic syndrome (5). It is also regarded as a major cause of cirrhosis of the liver of unknown cause (13). It is an established concept that hepatocellular disease, such as hepatocellular carcinoma and adenoma, cause platelet dysfunction and bleeding (14-16). It is also accepted that FLD, including the nonalcoholic form, is accompanied by platelet abnormalities through down-regulation of the ability of liver cells to produce thrombopoietin, which is an essential factor for platelet production. However, the influence of nonalcoholic FLD on platelet function and bleeding is not well understood (17). In the present study, we therefore examined the influence of nonalcoholic FLD on platelet function including platelet aggregation activity and bleeding using a rat experimental system.

In the first experiments, we examined whether oral administration of DAB could induce hepatopathy, especially nonalcoholic FLD, in rats. Histological observation of liver tissues stained with hematoxylin-eosin revealed the presence

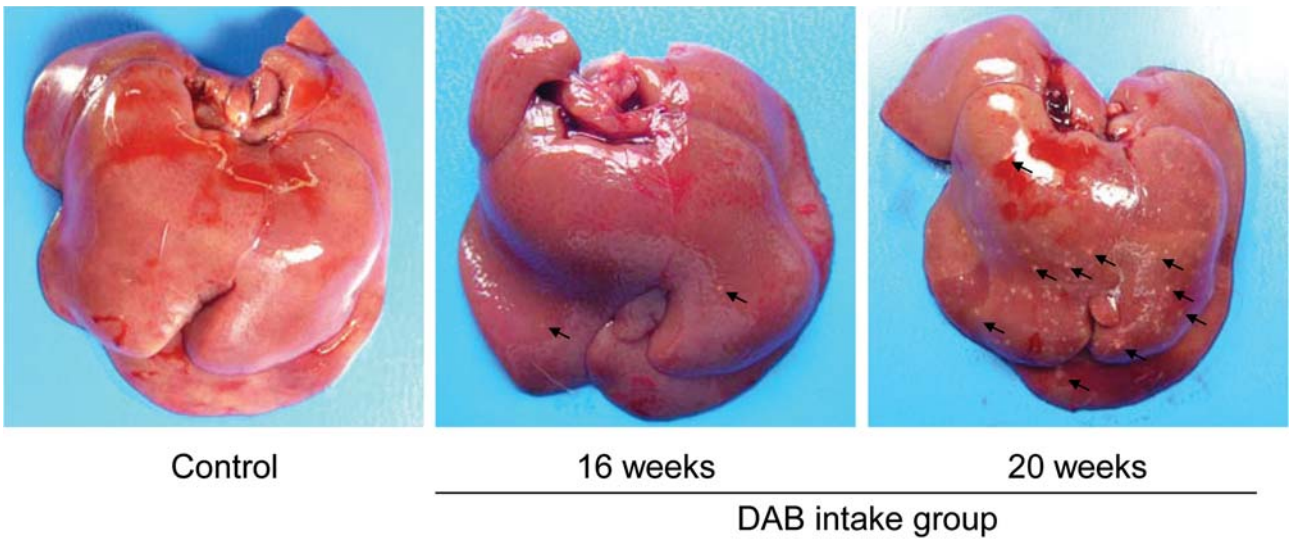

Figure 2. *Autopsy findings. F344 rats were fed powder diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (DAB) for 8, 12, 16 and 20 weeks. The arrows indicative degenerative areas.*

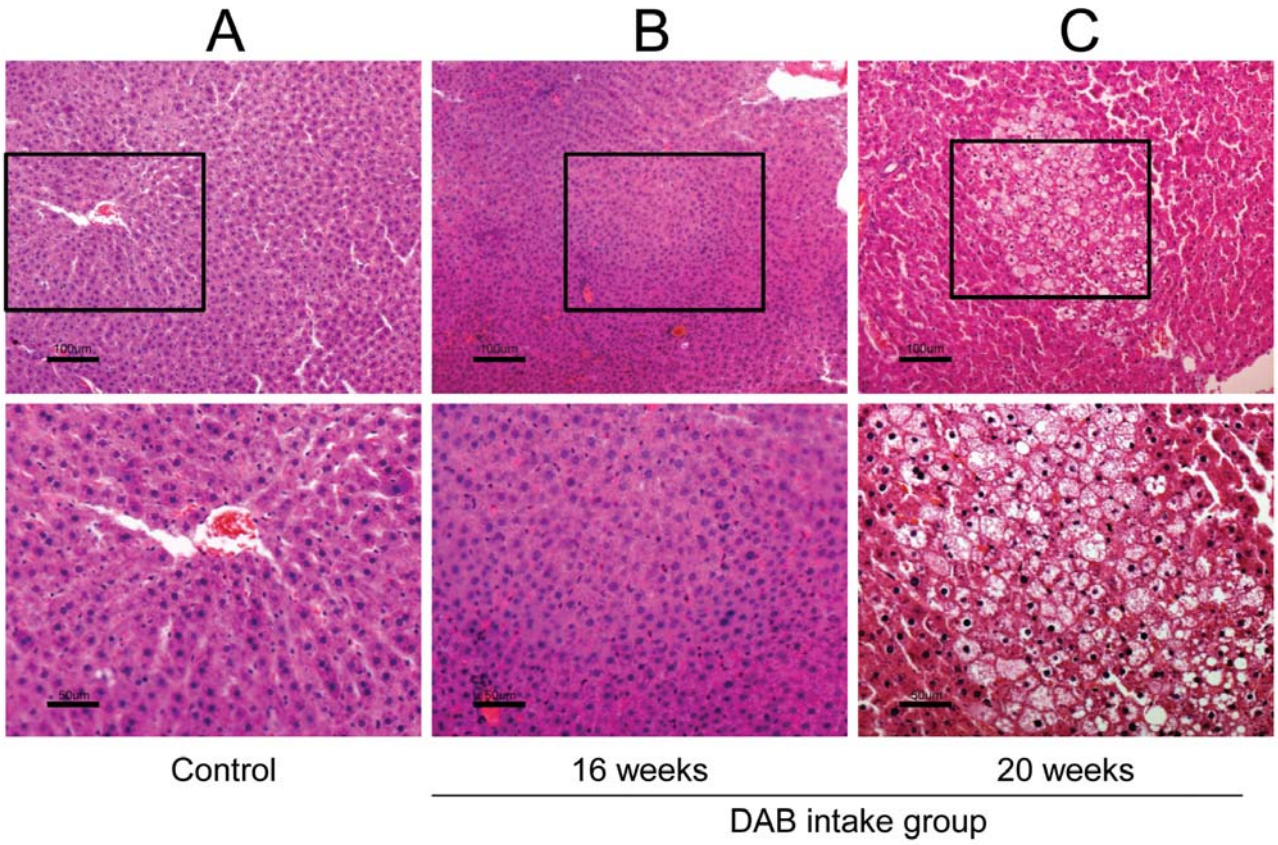

Figure 3. *Histological findings (hematoxylin-eosin staining). F344 rats were fed powder diet alone (A) or containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (DAB) for 8, 12, 16 (B) and 20 (C) weeks (w). The tissue of DAB-fed animals for 8 and 12 weeks did not have a morbid change (data not shown). The liver of the group fed DAB for 20 weeks exhibited wide areas of fatty degeneration. Upper panel: scale bars=100 μm; lower panel, higher magnification of area shown in the upper panel: scale bars=50 μm.*

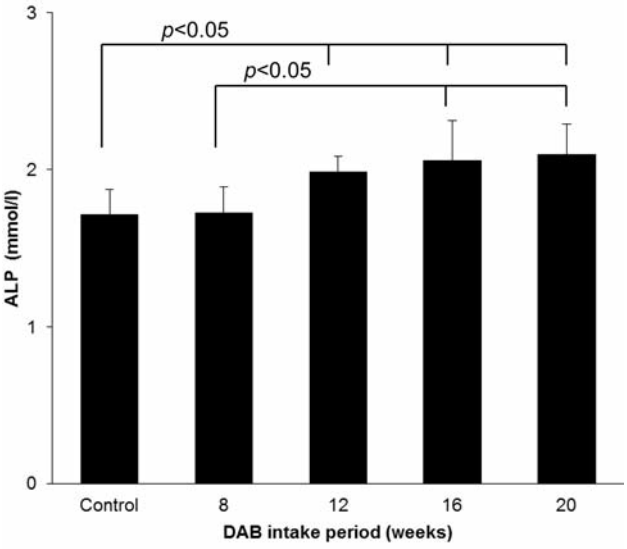

Figure 4. *Influence of 3'-methyl-4-dimethylaminoazobenzene (DAB) diet on alkaline phosphatase (ALP) levels in plasma. F344 rats were fed powder diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (DAB) for 8, 12, 16 and 20 weeks. Plasma ALP levels were examined by the ALP assay kit. The data are expressed as the mean±SD of 7 rats.*

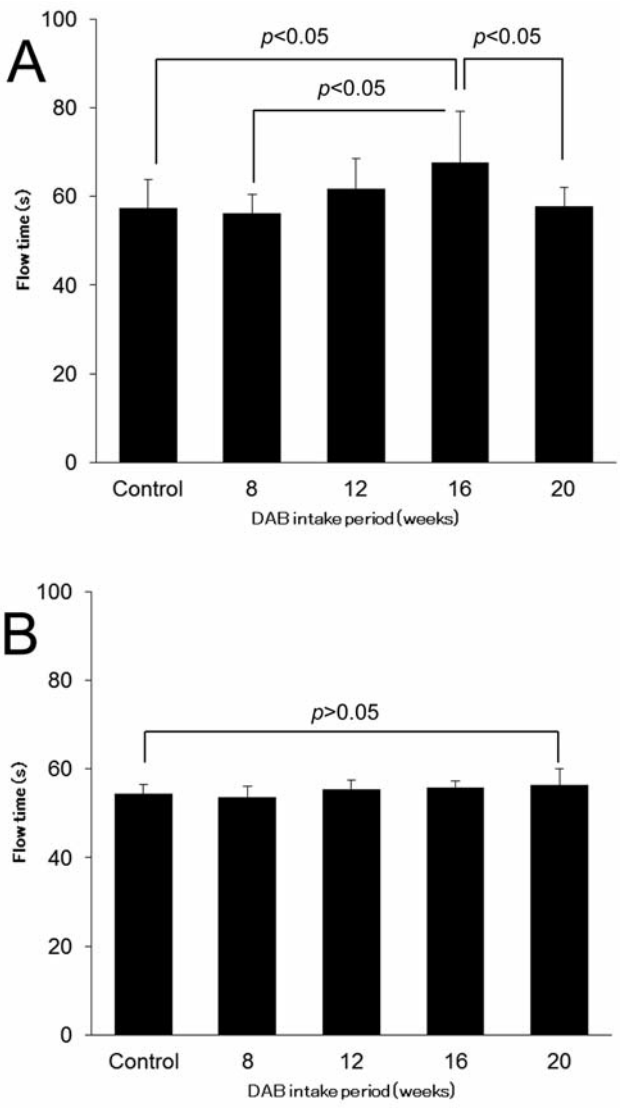

Figure 5. *Influence of 3'-methyl-4-dimethylaminoazobenzene (DAB) diet on blood fluidity. F344 rats were fed powder diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (DAB) for 8, 12, 16 and 20 weeks. The fluidity of blood treated with either heparin (A) or EDTA (B) was examined by an MC-FAN system. The data are expressed as the mean±SD of 7 rats.*

of balooning degeneration and of fatty cyst when the tissues were obtained from rats fed DAB for more than 16 weeks. These histological changes clearly showed that the hepatic tissue had undergone repeated cycles of destruction and regeneration. Furthermore, levels of ALP, which is the most important factor for assessment of hepatopathy (18), also increased in plasma obtained from long-term (for more than 16 weeks) DAB-fed rats as compared with that from control rats. These results may strongly suggest that an early hepatic disorder, which may be similar to the nonalcoholic FLD state, was developed in rats by feeding of the DAB-containing diet.

It is reported that oral administration of DAB to rats caused a decrease in the number of mitochondria in the liver (19). Furthermore DAB reduced the activity of several types of mitochondrial enzymes (19), responsible for fatty metabolism, especially those for beta oxidation of fatty acids, indicating that long-term feeding of DAB down-regulates mitochondrial functions, especially fatty metabolism, and results in the development of nonalcoholic FLD-like states. The present results also suggest that the rat model used in this study provides a realistic experimental model for elucidating the mechanisms of development of human nonalcoholic FLD such as NASH and its treatment.

In the second part of our experiments, we examined the influence of early hepatic disorder on blood fluidity by using an MC-FAN. The present results clearly showed that the fluidity of blood obtained from long-term DAB-fed rats decreased as compared with that from controls when heparin but not EDTA-2K was used as anticoagulant. Sodium heparin combines with antithrombin III and inhibits thrombin activity, coagulation factor Xa and XIIa, indicating that it does not inhibit agglomeration of platelets directly in prevention of blood coagulation (20). On the other hand, EDTA-2K inhibits the combination of proteins with fat because EDTA-2K chelates calcium ionized in blood, indicating that blood fluidity observed with an MC-FAN system reflects platelet aggregation ability (20). Furthermore, the number of blood cells, especially red blood cells, also affects

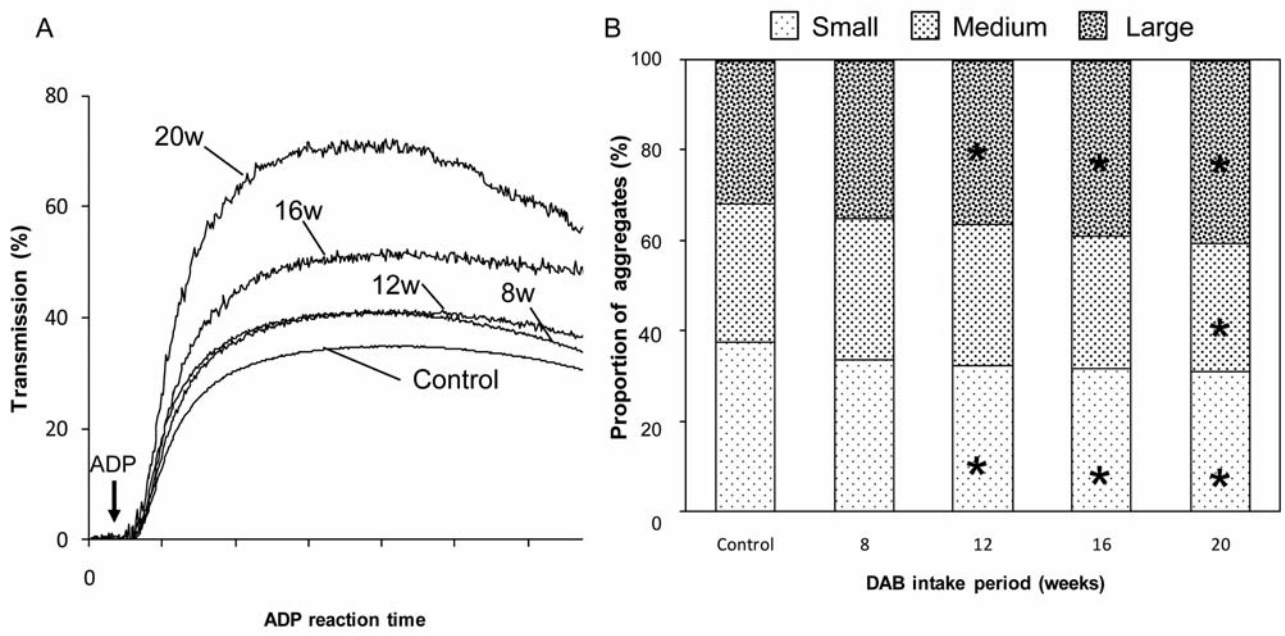

Figure 6. *Influence of 3'-methyl-4-dimethylaminoazobenzene (DAB) diet on platelet aggregation. F344 rats were fed powder diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (DAB) for 8, 12, 16 and 20 weeks (w). The platelet aggregation activity was examined by the light transmission method (A) and the light scattering method (B). The data are expressed as the mean±SD of 7 rats. *p<0.05 vs. control.*

blood fluidity and a decrease in the number of red blood cells results in an increase in blood fluidity (10, 21-23). Judging from these reports, the present results reasonably indicate that early hepatopathy reduced blood fluidity through enhancement of the ability of platelets for aggregation. This speculation may be supported by the observation that the platelet aggregation ability of the blood obtained from DAB-fed rats was higher as compared with that from control rats.

In conclusion, the present results suggest that the measurement of blood fluidity, as well as the assay of platelet aggregation ability, is useful in order to predict the appearance of thrombus in hepatopathy. Therefore, we think that these results may contribute to early diagnosis of thrombus disseminated intravascular coagulation of early hepatopathy, including NASH.

References

1 Eguchi Y, Mizuta T, Sumida Y, Ishibashi E, Kitajima Y, Isoda H, Horie H, Tashiro T, Iwamoto E, Takahashi H, Kuwashiro T, Soejima S, Kawaguchi Y, Oda Y, Emura S, Iwakiri R, Ozaki I, Eguchi T, Ono N, Anzai K, Fujimoto K and Koizumi S: The pathological role of visceral fat accumulation in steatosis, inflammation, and progression of nonalcoholic fatty liver disease. J Gastroenterol *46(1)*: 70-78, 2011.

2 Hotamisligil GS, Shargill NS and Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science *259*: 87-91, 1993.

3 Postic C and Girard J: Contribution of *de novo* fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest *113(3)*: 829-838, 2008.

4 Angulo P: Nonalcoholic fatty liver disease. N Engl J Med *346(16)*: 1221-1231, 2002.

5 Ota T, Takamura T, Kurita S, Matsuzawa N, Kita Y, Uno M, Akahori H, Misu H, Sakurai M, Zen Y, Nakanuma Y and Kaneko S: Insulin resistance accelerates a dietary rat model of nonalcoholic steatohepatitis. Gastroenterology *132(1)*: 282-293, 2007.

6 Miyamoto K, Hiramatsu K, Ohtaki Y, Kanitani M, Nomura M and Aburada M: Effects of gomisin A on the promotor action and serum bile acid concentration in hepatocarcinogenesis induced by 3'-methyl-4-dimethylamino-azobenzene. Biol Pharm Bull *18(10)*: 1443-1445, 1995.

7 Yang M, Yamamura K, Yan Y, Fukamachi Y, Hirano T, Kawamoto T and Higashi K: Studies on the microsomal cytochrome P450s in the livers of carcinogen-resistant and sensitive rats during long-term administration of 3'-methyl-4-dimethylaminoazobenzene. Journal of University of Occupational and Environmental Health *19(4)*: 277-286, 1997.

8 Asano K, Satoh K, Hosaka M, Arakawa H, Inagaki M, Hisamitsu T, Maeda M, Kochi M and Sakagami H: Production of hydrogen peroxide in cancerous tissue by intravenous administration of sodium 5,6-benzylidene-L-ascorbate. Anticancer Res *19*: 229-236, 1999.

9 Kikuchi Y, Sato K and Ohki H: Optically accessible microchannels formed in a single-crystal silicon substrate for studies of blood rheology. Microvasc Res *44*: 226-240, 1992.

10 Seki K, Sumino H and Murakami M: Study on blood rheology measured by MC-FAN. Rinsho Byori *51*: 770-775, 2003 (in Japanese).

11 Matsuno H, Tokuda H, Ishisaki A, Zhou Y, Kitajima Y and Kozawa O: P2Y12 receptors play a significant role in the development of platelet microaggregation in patients with diabetes. J Clin Endocrinol Metab *90*: 920-927, 2005.
12 Yamamoto T, Kamei M, Yokoi N, Yasuhara T, Tei M and Kinoshita S: Platelet aggregates in various stages of diabetic retinopathy: evaluation using the particle-counting light-scattering method. Graefes Arch Clin Exp Ophthalmol *243*: 665-670, 2005.
13 Sanyal AJ. American Gastroenterological Association: AGA technical review on nonalcoholic fatty liver disease. Gastroenterology *123(5)*: 1705-1725, 2002.
14 Boye NP, Nordöy A and Gjone E. Splenectomy in active chronic hepatitis. Scand J Gastroenterol *7(8)*: 747-750, 1972.
15 Ikeda K, Saitoh S, Kobayashi M, Suzuki Y, Tsubota A, Suzuki F, Arase Y, Murashima N, Chayama K and Kumada H: Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical discriminant function using common laboratory data. Hepatol Res *18(3)*: 252-266, 2000.
16 Witters P, Freson K, Verslype C, Peerlinck K, Hoylaerts M, Nevens F, Van Geet C and Cassiman D: Blood platelet number and function in chronic liver disease and cirrhosis. Aliment Pharmacol Ther *27(11)*: 1017-1029, 2008.
17 Yoneda M, Fujii H, Sumida Y, Hyogo H, Itoh Y, Ono M, Eguchi Y, Suzuki Y, Aoki N, Kanemasa K, Imajo K, Chayama K, Saibara T, Kawada N, Fujimoto K, Kohgo Y, Yoshikawa T and Okanoue T; Japan Study Group of Nonalcoholic Fatty Liver Disease. Platelet count for predicting fibrosis in nonalcoholic fatty liver disease. J Gastroenterol *46(11)*: 1300-1306, 2011.
18 Kawai H, Kudo N, Kawashima Y and Mitsumoto A: Efficacy of urine bile acid as a non-invasive indicator of liver damage in rats. J Toxicol Sci *34(1)*: 27-38, 2009.
19 Schneider WC, Hogeboom GH, Shelton E and Striebich MJ: Enzymatic and chemical studies on the livers and liver mitochondria of rats fed 2-methyl- or 3-methyl-4-dimethylaminoazobenzene. Cancer Res *13(3)*: 285-288, 1953.
20 Surin WR, Barthwal MK and Dikshit M: Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. Thromb Res *122(6)*: 786-803, 2008.
21 Ujiie H, Kawasaki M, Suzuki Y and Kaibara M: Influence of age and hematocrit on the coagulation of blood. J Biorheology *23(2)*: 111-114, 2009.
22 Ariyoshi K, Maruyama T, Odashiro K, Akashi K, Fujino T, Uyesaka N and Circ J: Impaired erythrocyte filterability of spontaneously hypertensive rats: investigation by nickel filtration technique. Circ J *74(1)*: 129-136, 2010.
23 Sano M, Ichihashi Y, Aoto A, Tanaka M, Kojima T and Morita T: Platelet aggregation measurement using MCFAN. Medicine Biol *151(11)*: 538-545, 2010.

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