

## Changes in Coagulative and Fibrinolytic Activities in Triton WR-1339-Induced Hyperlipidemia in Rats

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**Abstract**—Changes in coagulative and fibrinolytic activities were studied in rats with hyperlipidemia induced by Triton WR-1339 (T-WR). After intravenous injection of T-WR (150, 200 or 300 mg/kg) into S.D. rats, dose-related increases in plasma lipids (total cholesterol, triglyceride, free cholesterol and phospholipid) were observed. In hyperlipidemic rats that received 300 mg/kg of T-WR, decreases in red blood cell count and Hb value were found. Significant increases in the *ma* value of the thromboelastogram and the fibrinogen level were observed in these T-WR treated rats. The  $\alpha_2$ -plasmin inhibitor activity was found to decrease dose-relatedly. These results indicate that T-WR induced hyperlipidemia in rats is accompanied with an increase in coagulative activity and an indirect enhancement of fibrinolytic activity.

Several risk factors have been associated with the development of atherosclerosis and its clinical complications. These factors include elevated plasma lipid, smoking, diabetes, hypertension and genetic predisposition. Especially, a fat- and cholesterol-rich diet is suggested to be a major factor leading to atherosclerosis (1). Some clinical reports (2, 3) discussed the risk of thrombosis in atherosclerotic cases. Thereafter, atherosclerosis has been considered to be the underlying disease of thrombosis because of its hypercoagulative activity. Furthermore, the increase in coagulative activity and the decrease in fibrinolytic activity have been believed to occur in patients with some kind of atherosclerosis, although direct evidence has been lacking (4). However, the mechanism of thrombus formation in hyperlipidemia and atherosclerosis has not been completely understood. Especially, the relationship between plasma lipid and the blood coagulative and fibrinolytic activity in patients and experimental animals with hyperlipidemia has only been poorly understood. Revealing the cause and/or the mechanisms of atherosclerosis development in hyperlipidemia will be very important for finding new anti-

atherosclerotic drugs. Since it has been discovered that Triton WR-1339 (T-WR) rapidly causes hyperlipidemia in experimental animals (5, 6), the present investigation is designed to examine the blood coagulative and fibrinolytic activity in T-WR induced hyperlipidemic rats.

### Materials and Methods

#### Animal and drug administration

Male Sprague-Dawley rats, 6 weeks of age at the start of the experiments, were fed with a stock diet (MF, Oriental Kobo Co., Ltd.) and tap water *ad libitum* under standard laboratory conditions ( $21 \pm 2^\circ\text{C}$ ,  $55 \pm 15\%$  humidity). Rats were divided into four groups: saline control group and T-WR (150, 200 and 300 mg/kg)-treated groups.

Triton WR-1339 (p-isooctylpolyoxy-ethyl-enphenol formaldehyde polymer) was purchased from Nakai Chemical Industries, Ltd. Each animal intravenously received T-WR or saline and was fasted for 24 hr. We determined all items for the study 24 hr after T-WR administration.

#### Blood collection and preparation

Blood specimens were taken from the inferior vena cava with a plastic syringe and

silicon-coated needle under pentobarbital anaesthesia (40 mg/kg, i.p.). We used whole blood to determine the thromboelastogram (TEG).

Citrated plasma was prepared as follows: whole blood from rats was mixed with 3.2% sodium citrate solution in a volume ratio of nine to one, and then it was centrifuged at 3000 r.p.m. for 15 min at 4°C. The supernatant was used as platelet-poor plasma.

#### Chemical assays

Total cholesterol (TC) in the plasma was determined by a colorimetric method (Cholesterol-B Test) (7). Phospholipid (PL) in the plasma was determined enzymatically (Phospholipid-B Test) (8). Free cholesterol (FC) and triglyceride (TG) in the plasma were determined enzymatically (Free cholesterol-C Test, Triglyceride-G Test) (9, 10). These assays were performed using commercial kits (Wako Pure Chemical Ind., Ltd., Tokyo).

#### Blood examination

Citrated whole blood was analyzed for platelet, white blood cell (WBC) and red blood cell (RBC) counts and the values of the hematocrit (Ht) and hemoglobin (Hb) using automated equipment (Coulter Counter, S-plus size distribution, Coulter Electronics, Inc.).

#### Assay of coagulative activity

**Thromboelastogram (TEG):** As shown in our previous paper (11), the total coagulative process was depicted by thromboelastography (Clot-tracer TE-30, Erma Co., Ltd.). From the thromboelastogram obtained with 0.35 ml of whole blood, *r*, *k* and *ma* values were determined (Fig. 3A). *R* stands for the reaction time that elapsed from the beginning of blood collection to the time when the amplitude reached 1 mm. *K* stands for the clot formation time that elapsed from the time at which the *r* value was determined to the time when the amplitude reached 20 mm. The *ma* stands for the maximum amplitude.

**Prothrombin time (PT) and partial thromboplastin time (PTT):** PT was measured using Simplastin (Warnar-Lambert Co. Ltd.) by a modified one-stage method. Simplastin contains a tissue thromboplastin reagent (rabbit brain and lung),  $\text{CaCl}_2$  and NaCl. PTT was measured using Platelin (Warnar-Lambert

Co. Ltd.) by the method of Langdell et al. (12). Platelin contains plasma thromboplastin reagent (rabbit brain phospholipid) and NaCl. Measurement of PT and PTT was conducted using Clot-Digitim TE-20 (Erma Optical Works, Ltd.).

**Fibrinogen level:** The fibrinogen level was measured by the method of Tomikawa (13). Citrated plasma was mixed with  $\text{CaCl}_2$  and tranexamic acid (Sigma Chemical Co.), and the mixture was incubated at 37°C. After removal of the nonclottable proteins from the diluted plasma clot by centrifugation, the protein content of the fibrin precipitate was determined by the method of Lowry et al. (14).

**Factor XIII:** We followed the conventional method using a commercial kit (F. XIII, Iatron Laboratories, Inc.). The activities of factor XIII were determined on the basis of the intensities of fluorescence from the dansylcadaverine complex by a fluorescence spectrofluorometer (Hitachi 650-10M Fluorescence spectrophotometer).

**Antithrombin III (ATIII):** ATIII activity was determined by the method using synthetic chromogenic substrates (Tosyl-Gly-Pro-Arg-pNA; Chromolate ATIII, Iatron Laboratories, Inc.).

#### Assay of fibrinolytic activity

**Plasminogen (PLG):** PLG assay was conducted by the chromogenic method using S-2251 as a substrate (Kabi Diagnostica, Daiichi Chemical Pharmacy Co., Ltd.). We used urokinase (Mochida Pharmaceutical Co., Ltd.) as the PLG activator because the PLG of the rat and mouse was activated by urokinase but not by streptokinase (15).

**$\alpha_2$ -Plasmin inhibitor ( $\alpha_2$ -PI):** Activity of  $\alpha_2$ -PI was determined by a method using the synthetic chromogenic substrate S-2251.

#### Statistical analysis

Results were expressed as the mean  $\pm$  S.D. from 7 to 9 rats per group. Statistical significance was evaluated using Student's *t*-test.

### Results

#### Plasma lipids

Figure 1 shows the changes in the plasma lipids in rats treated with 150, 200 or 300 mg/kg of T-WR. We can observe the dose-

related increase in plasma TC, FC, TG and PL at 24 hr after intravenous injection of T-WR. The correlation coefficients of plasma TC, FC, TG and PL for T-WR doses are 0.8997, 0.8288, 0.8284 and 0.8432, respectively.

### Blood examinations

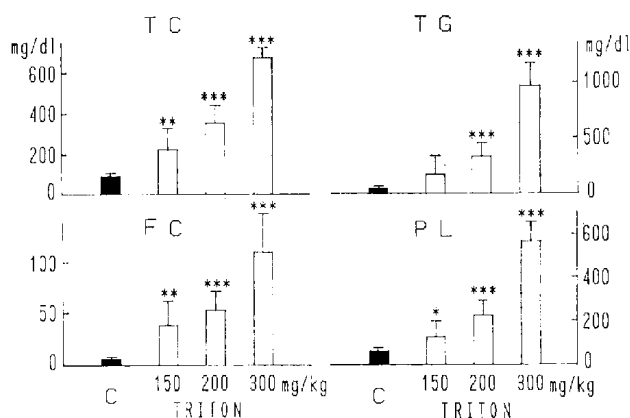
Table 1 shows the changes in the counts of various blood cells and Hb and Ht values in rats with hyperlipidemia induced by T-WR. RBC counts decreased significantly in rats treated with 300 mg/kg of T-WR, but platelet counts did not change. WBC counts decreased in rats treated with 150 mg/kg of T-WR and increased in rats treated with 300 mg/kg of the same compared with that of control rats. The Hb value of rats injected

with T-WR decreased dose-relatedly compared with that of the saline control. The correlation coefficient of the Hb value for T-WR doses was  $-0.7454$ .

### Coagulative and fibrinolytic activity

**TEG:** Figure 2 shows the  $r$ ,  $k$  and  $ma$  values of TEG after T-WR treatment. No significant effects on  $r$  and  $k$  values were observed, but an apparent increase in  $ma$  value was observed in T-WR treated rats (Fig. 2 and Fig. 3, B). On the other hand, we observed abnormal enhancement of fibrinolysis in some of the rats treated with 300 mg/kg of T-WR in the TEG figures (Fig. 3, C).

**PT, PTT and ATIII:** Table 2 shows the effect of T-WR on PT, PTT and ATIII activity. PT shortened slightly ( $P<0.1$ ) in rats treated



**Fig. 1.** Mean concentrations of total plasma cholesterol (TC), triglycerides (TG), free cholesterol (FC) and phospholipid (PL) in T-WR treated rats. Rats were intravenously injected with saline or with 150, 200 or 300 mg/kg of T-WR and fasted 24 hr. C: saline control. Plasmalipid concentrations are shown in mg/dl, and each value represents the mean  $\pm$  S.D. of 7–9 specimens. \*\*\*, \*\*, \*: significant difference from the saline control value with  $P<0.001$ ,  $P<0.01$ ,  $P<0.05$ , respectively (Student's  $t$ -test).

**Table 1.** The counts of various blood cells and hemoglobin (Hb) and hematocrit (Ht) values at 24 hr after Triton injection in rats

	Control	Triton (mg/kg)		
		150	200	300
PLA ( $\times 10^5/\mu\text{l}$ )	9.75 $\pm$ 1.55	10.88 $\pm$ 1.26	10.54 $\pm$ 1.53	9.40 $\pm$ 1.64
WBC ( $\times 10^3/\mu\text{l}$ )	6.71 $\pm$ 0.92	5.08 $\pm$ 0.59**	7.17 $\pm$ 0.78	9.77 $\pm$ 2.64*
RBC ( $\times 10^6/\mu\text{l}$ )	6.71 $\pm$ 0.32	6.55 $\pm$ 0.26	6.84 $\pm$ 0.54	5.90 $\pm$ 0.47**
Hb (g/dl)	13.8 $\pm$ 0.51	13.3 $\pm$ 0.47	12.9 $\pm$ 0.95	11.8 $\pm$ 0.66**
Ht (%)	38.4 $\pm$ 1.62	39.3 $\pm$ 2.32	40.0 $\pm$ 3.27	35.9 $\pm$ 3.16

PLA=platelet, WBC=white blood cell, RBC=red blood cell, Hb=hemoglobin, Ht=hematocrit. Each value represents the mean  $\pm$  S.D. of 7 specimens. \*\*, \*: significant difference from the saline-control value with  $P<0.01$ ,  $P<0.05$ , respectively (Student's  $t$ -test).

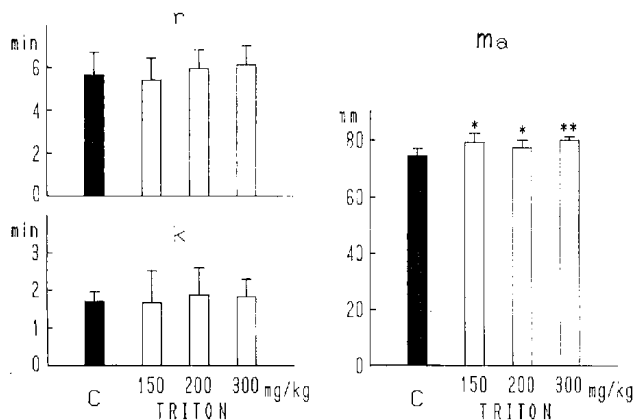


Fig. 2. Changes in *r*, *k* and *ma* values on the thromboelastogram in the T-WR induced hyperlipidemic rats. *r* and *k* values are shown in min, and *ma* value is shown in mm. C: saline control. Each value represents the mean  $\pm$  S.D. of 7–8 specimens. \*\*\*: significant difference from the saline control value with  $P < 0.01$ ,  $P < 0.05$ , respectively (Student's *t*-test).

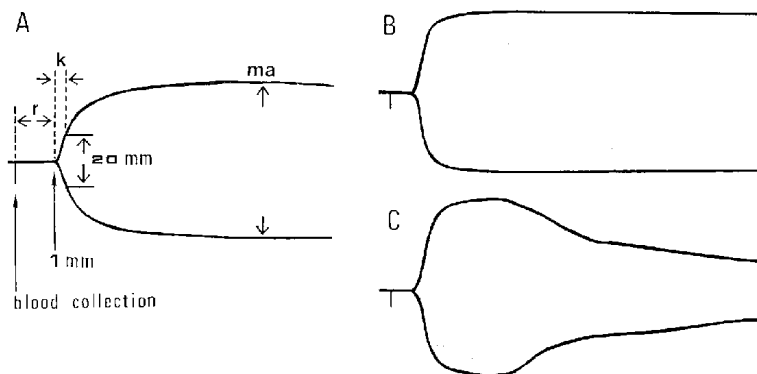


Fig. 3. The definition of *r*, *k* and *ma* value on the thromboelastogram (TEG) and typical examples of TEG in T-WR induced hyperlipidemic rats. A: saline control. B and C: 300 mg/kg of T-WR. Different patterns on the TEG were obtained by thromboelastography in the rats treated with 300 mg/kg of T-WR. B shows an enhancement of coagulative activity, and C shows an enhancement of fibrinolytic activity.

Table 2. Prothrombin time (PT), partial thromboplastin time (PTT) and antithrombin III (ATIII) in Triton-induced hyperlipidemic rats

	Control	Triton (mg/kg)		
		150	200	300
PT (sec)	10.0 $\pm$ 0.9	9.5 $\pm$ 0.9	9.3 $\pm$ 0.8	9.3 $\pm$ 0.5
PTT (sec)	36.7 $\pm$ 7.1	38.8 $\pm$ 5.9	36.3 $\pm$ 6.4	34.1 $\pm$ 5.6
ATIII (%)	100.4 $\pm$ 20.3	102.3 $\pm$ 8.2	113.2 $\pm$ 10.2	111.8 $\pm$ 15.0

PT and PTT are shown in sec, and the activities of ATIII are shown as a ratio compared with those in normal human plasma. Each value represents the mean  $\pm$  S.D. of 9 specimens.

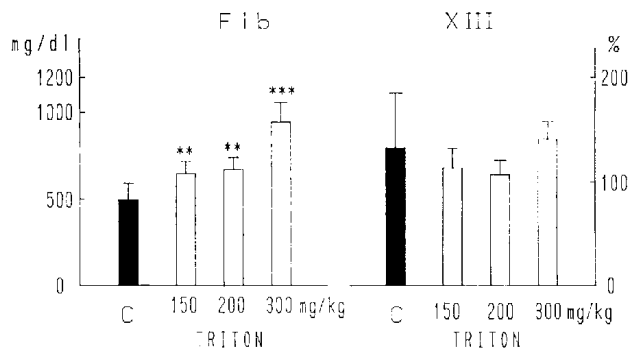


Fig. 4. Changes in fibrinogen (Fib) level and the activity of factor XIII in the T-WR induced hyperlipidemic rats. Fib levels are shown in mg/dl, and the activities of factor XIII are shown as intensities of dansylcadaverine complex fluorescence determined by a fluorescence spectrofluorometer. C: saline control. Each value represents the mean  $\pm$  S.D. of 7–8 specimens. \*\*\*, \*\*: significant difference from the saline control value with  $P < 0.001$ ,  $P < 0.01$ , respectively (Student's *t*-test).

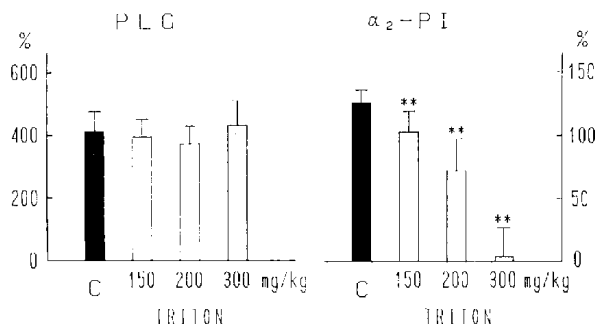


Fig. 5. Changes in plasminogen (PLG) level and the activity of  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) in T-WR induced hyperlipidemic rats. PLG levels and  $\alpha_2$ -PI activities are shown as a ratio compared with those in normal human plasma. C: saline control. Each value represents the mean  $\pm$  S.D. of 7–9 specimens. \*\*: significant difference from the saline control value with  $P < 0.01$  (Student's *t*-test).

with 200 and 300 mg/kg of T-WR, but PTT and ATIII activity showed no significant changes in all T-WR treated rats.

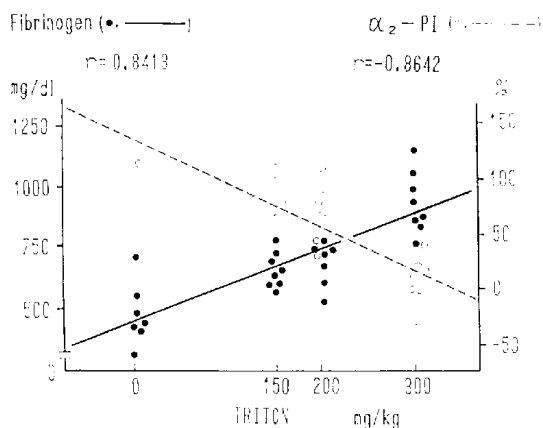
**Fibrinogen and factor XIII:** Figure 4 shows the change in fibrinogen content and the activity of factor XIII 24 hr after T-WR treatment. Fibrinogen level increased dose-relatedly after T-WR treatment. We can see no apparent effect of T-WR on XIII activity because of the wide variation of control values in the saline-treated group.

**PLG and  $\alpha_2$ -PI:** Figure 5 shows the change of PLG content and  $\alpha_2$ -PI activity in the hyperlipidemic rats induced by T-WR. We can not find any significant changes in PLG level, but  $\alpha_2$ -PI activity remarkably decreased with the increase in the doses of T-WR.

Figure 6 shows the interrelationship between the fibrinogen level and  $\alpha_2$ -PI activity in rats with hyperlipidemia induced by T-WR. The correlation coefficient between the doses of T-WR and fibrinogen levels was 0.8413, and that between the doses of T-WR and  $\alpha_2$ -PI activity was  $-0.8642$ . In hyperlipidemic rats induced by T-WR, there was an inter-related increase in fibrinogen content with a remarkable decrease in  $\alpha_2$ -PI activity.

## Discussion

The nonionic surfactant, T-WR, has been used in lipid metabolism research to inhibit the removal of lipoprotein from the circulation (16). It is convenient to use a T-WR induced hyperlipidemia model, because it can



**Fig. 6.** The interrelation between fibrinogen level and  $\alpha_2$ -PI activity in T-WR induced hyperlipidemic rats.  $r$ =correlation coefficient.

produce dose-related hyperlipidemia in a short period. In the present study, significant and positive linear correlations between T-WR doses and plasma lipids were obtained. The mechanisms by which T-WR causes hyperlipidemia include the following: inhibition of lipolysis of TG-rich lipoprotein (17), stimulation of hepatic cholesterol synthesis (18, 19), and probable efflux of cholesterol from body tissues (including liver) into the circulation. The detergent forms a coating around the lipoprotein that protects very-low-density lipoproteins (VLDL) from hydrolysis by lipoprotein lipase (20, 21). As to the increase in TG and VLDL, the T-WR induced hyperlipidemia in rats can be considered to resemble the IV type hyperlipidemia in man.

The major findings in this study are the increase in *ma* value of TEG and fibrinogen levels and the decrease in  $\alpha_2$ -PI activity. TEG is used to determine the entire mobilization of coagulative and fibrinolytic activity (22). The  $r$  value contributed to the thromboplastin formation time, and the  $k$  value was equivalent to the thrombin formation time. The *ma* value indicated the strength of the clot; that is, the platelet numbers and function and fibrinogen content could be judged from it. In this experiment, the increase in *ma* value in T-WR-treated rats is considered to be due to the increase in fibrinogen contents.

Especially, a positive relationship between

plasma lipids and fibrinogen levels was obtained in T-WR induced hyperlipidemic rats. Ishida et al. (23) reported that both fibrin and fibrinogen stimulated the proliferation of rabbit aortic smooth muscle cells in culture. In addition, Kadish et al. observed that the normal monolayer architecture of cultured vascular endothelium becomes rapidly disorganized after contact of the cell layer with a fibrin clot (24). They proposed that mural fibrin in vivo may produce a disorganized endothelium which induces further fibrin deposition and platelet aggregation in cultured bovine aortic endothelium (25). From our data, the following process is suggested. The increase in fibrinogen level induces the enhancement of fibrin formation. The increased fibrin can penetrate into the endothelium and stimulate the proliferation of the smooth muscle cells. A denuding injury to the endothelium in vivo leads to fibrin deposition. Furthermore, the indirect elevation of fibrinolysis due to the decrease of  $\alpha_2$ -PI activity might also enhance the increase in vascular permeability in the rats with T-WR induced hyperlipidemia. Therefore, many lipid particles can easily penetrate into the endothelial cells, and these phenomena will induce the first stage of atherosclerosis in T-WR induced hyperlipidemia. However, it is necessary to examine coagulative and fibrinolytic activities during a long experimental period to demonstrate these hypothesis.

There have been a few reports about fibrinolysis in hyperlipidemia. Nishino reported that fibrinolytic activity decreased during the initial 2 weeks after a cholesterol-rich diet in experimental atherosclerosis using rabbits (26). On the other hand, Kobayashi et al. (27) examined blood coagulation-fibrinolytic factors and serum lipids in aged patients with old cerebral thrombosis (average age: 72.4 years). They found that the patients had significantly higher fibrinogen levels, but their  $\alpha_2$ -PI levels were significantly lower. In the present study of T-WR induced hyperlipidemia, the decrease in  $\alpha_2$ -PI activity was observed, and some rats with 300 mg/kg T-WR treatment manifest abnormal enhancement of fibrinolysis in the figures of TEG. These results were

in agreement with those obtained by Kobayashi et al. (27), but no papers on blood coagulative and fibrinolytic activity in type IV hyperlipidemia in man were found. In a study using plasma from a patient with congenital  $\alpha_2$ -PI deficiency, fibrinolysis can be induced by fibrin itself without activation of the coagulation cascade and the induction of fibrinolysis is efficiently blocked by  $\alpha_2$ -PI (28). We had already determined that T-WR had no direct effect on the assay for  $\alpha_2$ -PI activity (data not shown). Therefore, there might be a decrease in  $\alpha_2$ -PI activity in T-WR induced hyperlipidemia that inhibits the hypercoagulation due to the increase in fibrinogen level. However, it remains unsettled whether this occurs in T-WR induced hyperlipidemic rats alone or not.

Various hypotheses have been proposed for explaining the mechanisms of coagulation pathway activation at the site of vascular injury. Upon injury of endothelial or smooth muscle cells, tissue thromboplastin can activate both the extrinsic and intrinsic pathways of coagulation (29). However, in our experiments in hyperlipidemic rats, the extrinsic pathway of coagulation was enhanced mildly, while the intrinsic pathway of coagulation showed no changes. Suehiro et al. reported that thrombi are likely to be formed in hyperlipidemia and that such thrombus formation is due largely to platelet hyperfunction (30). In this connection, it is worthwhile to examine platelet function in these hyperlipidemic rats. Such studies are now underway in our laboratory and the results will be reported soon.

In the present study, the RBC counts and Hb value in hyperlipidemic rats decreased after the injection of 300 mg/kg of T-WR. In fact, we observed hematuria, hamafecia and hemolysis in some rats treated with 300 mg/kg of T-WR. Scanu et al. reported that the osmotic fragility of the red cells increased proportionally to the amount of T-WR employed (31). Since T-WR as a detergent has a hemolytic side effect, large doses of T-WR might induce the decrease in RBC counts and Hb values. The changes in WBC counts showed contrasting results depending on T-WR doses. That is, small doses of T-WR induced a decrease in WBC counts,

while large doses of the same drug induced an increase. This decrease of WBC counts in the mild hyperlipidemia induced by 150 mg/kg of T-WR is considered to result from the consumption of the cells to clean up the surplus lipid in the circulation. The increase of WBC counts in rats with severe hyperlipidemia induced by 300 mg/kg of T-WR might be explained by an inflammatory reaction such as an acute phase reactive response. Furthermore, large amounts of T-WR may have altered the permeability of the vascular endothelium and/or the vascular smooth muscle through a direct combination of the detergent with the lipoprotein lipids of the cell membranes (31). These results indicate that the intravenous injection of 200 mg/kg of T-WR is suitable for the experimental model of hyperlipidemia in rats. On the basis of the results of the present study using this experimental model, the increase in fibrinogen level and the decrease in  $\alpha_2$ -PI activity may be useful for making a diagnosis and predicting the prognosis of hyperlipidemia, except for the increase in plasma lipids.

The present investigation has shown that there are increases in the ma value of TEG and fibrinogen level and a decrease in  $\alpha_2$ -PI activity in T-WR induced hyperlipidemic rats. The increase in coagulative activity and indirect enhancement of fibrinolysis are strongly suggested to induce the initiation and the development of the atherosclerosis in hyperlipidemic rats.

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