

# Effects of Fermented Rice Vinegar Kurozu and Its Sediment on Inflammation-Induced Plasminogen Activator Inhibitor 1 (PAI-1) Increase

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## Abstract

Kurozu is a traditional black rice vinegar in Japan. Kurozu-moromi is the solid sediment that arises during the production of Kurozu. Kurozu is thought to have blood thinning and antithrombotic activities but a specific effect has not been proven. Plasma plasminogen activator inhibitor 1 (PAI-1) inhibits fibrinolysis and when elevated, it is a risk factor for the development of thrombotic diseases. Here, we examined the effects of a diet containing Kurozu and Kurozu-moromi on LPS (lipopolysaccharide)-induced increases in plasma PAI-1 levels in mice and on TNF- $\alpha$  (tumor necrosis factor  $\alpha$ ) increased PAI-1 production in the medium of a cultured endothelial cell line. Mice were fed with a diet containing 0.25% (w/w) concentrated Kurozu or 0.5% (w/w) Kurozu-moromi for four weeks, and then subcutaneously injected with 0.015 mg/kg of LPS in saline and sacrificed three hours later. Orally administered concentrated Kurozu and Kurozu-moromi significantly suppressed the LPS-induced increase in PAI-1 antigen and its activity in mouse plasma. The ethanol extract of Kurozu-moromi inhibited TNF- $\alpha$  induced increases of PAI-1 in the culture medium of the EA.hy926 endothelial cell line. The present findings showed that Kurozu and Kurozu-moromi contain natural products that decrease thrombotic tendencies by decreasing PAI-1 production in inflammatory states and have potential as antithrombotic foodstuffs.

## Keywords

Kurozu, Mouse, Dietary Supplement, Endothelial Cells, Thrombosis

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### 1. Introduction

Plasminogen and its cleavage form, plasmin, are associated with the fibrinolytic system that is involved in fibrin degradation and clot removal. Plasmin formation is catalyzed by tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activators. Plasminogen activator inhibitor-1 (PAI-1) is the primary physiological inhibitor of t-PA and u-PA, and it plays an important role in the removal of thrombi from the vascular system under thrombotic conditions [1]. Increased plasma PAI-1 levels suppress the normal fibrinolytic system and lead to prothrombotic states. Thus, increased levels of plasma PAI-1 comprise a risk factor; serve as a marker for thrombotic diseases and onset of these diseases [1]. Levels of PAI-1 are elevated in various prothrombotic states such as hypertension, obesity, insulin resistance, diabetes and aging [2] [3] [4] [5]. Maintaining physiological plasma levels and activities of PAI-1 might thus represent a promising intervention for treating and preventing thrombotic diseases [6].

Endotoxins and proinflammatory cytokines play a central role in inflammation-induced prothrombotic conditions [7]. Namely, the acute phase alters the coagulation and fibrinolytic systems and frequently leads to a procoagulant state [8]. Plasminogen activator inhibitor 1 is produced as an acute-phase reactant. Thus, the suppression of increased PAI-1 expression during the acute phase would improve prothrombotic conditions in these situations. Some compounds from natural sources inhibit PAI-1 production *in vitro* and in experimental animals *in vivo*. Zhou *et al.* reported that salvianolic acid B attenuates PAI-1 production in human umbilical vein endothelial cells (HUVEC) incubated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) [9]. Lin *et al.* reported that green tea polyphenols inhibit PAI-1 expression and secretion in endothelial cells [10]. A citrus extract containing flavones represses PAI-1 expression in human colon fibroblasts [11]. We reported that xanthoangelol isolated from *Angelica keiskei* inhibits PAI-1 increases induced by lipopolysaccharide (LPS) in mouse plasma and by TNF- $\alpha$  in culture media of HUVEC [12]. We also showed that chrysin, a naturally occurring flavone, a type of flavonoid found in plants such as passion flower and chamomile, inhibits the TNF- $\alpha$  induced PAI-1 increase in the culture medium of endothelial cell line [13].

Kurozu is traditional Japanese black vinegar produced from unpolished rice. It is used in Japan as a seasoning agent or consumed as a health food. Kurozu is traditionally produced in the Kagoshima region of Japan by fermenting unpolished rice using several types of bacilli for over one year in earthenware jars. The matured liquid in the jar is filtered to produce Kurozu, and the remaining sediment at the bottom of jar is called Kurozu-moromi (KM). Kurozu contains

abundant amino acids, vitamins and organic acids [14] [15] [16] [17], whereas Kurozu-moromi is rich in organic materials, minerals, amino acids and so on [17].

Reports indicate that Kurozu has various health benefits, including anti-cancer activity against colon cancer cells cultured *in vitro* [18], and against colon cancer in an animal model [19]. Orally administered Kurozu decreases the size of adipocytes by inhibiting dietary fat absorption and reducing PPAR $\gamma$  and aP2 mRNA expression levels in rat adipocytes [14]. Kurozu has protective effects against experimental colitis induced by dextran sulfate sodium [20]. Kurozu has potent radical scavenging activity and suppress lipid peroxidation *in vitro* and in mouse skin [15] [21]. Feeding with a diet containing concentrated Kurozu ameliorates cognitive dysfunction and suppresses the accumulation of aggregated protein in the mouse brain [17]. Several studies have also described the effects of Kurozu-moromi on various diseases. Kurozu-moromi inhibits the tumor growth of Lovo cells in a mouse model *in vivo* [22] and inhibits the growth of hepatocellular carcinoma [23]. Both Kurozu and Kurozu-moromi protect against experimental colitis induced by dextran sulfate sodium [20].

The present study examines the effects of a diet containing Kurozu and Kurozu-moromi on LPS-induced increases in plasma PAI-1 levels in mice. We used lyophilized and 10-fold concentrated Kurozu (CK), and KM powder that was prepared from the mashed residue after Kurozu production. We then examined the effects of CK and Kurozu-moromi ethanol extract (KM-E) on the increase in PAI-1 produced induced by TNF- $\alpha$  in the culture medium of an endothelial cell line. The present findings showed that Kurozu and Kurozu-moromi protect against thrombotic tendencies by decreasing PAI-1 production in inflammatory states.

## 2. Materials and Methods

### 2.1. Materials

Kurozu and Kurozu-moromi were provided by Sakamoto Kurozu Inc., Fukuyama, Kagoshima, Japan. Ten-fold concentrated Kurozu (CK) was prepared by repeated lyophilization, which removes acetic acid. The chemical composition of CK was 82.6% water, 17.4% soluble solid 9.3% crude protein, 1.7% lactic acid, 0.6% direct reducing sugar, 0.01% acetic acid. Kurozu-moromi (Sakamoto Kurozu) was prepared from the mashed residue generated during Kurozu production. The residue was dried *in vacuo* at 110°C. The chemical composition of KM was 4% water, 12% crude protein, 23% lipid, 1% ash, and 60% carbohydrate.

Kurozu-moromi ethanol extract (KM-E) was prepared as follows. Ethanol (1500 mL) was added to Kurozu-moromi (500 g) and stirred for 15 min and then filtered. The residue was resuspended in ethanol (1500 mL) and the mixture was again stirred for 15 min. Both ethanol fractions were filtered, combined and evaporated. The yield of KM-E from 500 g of KM was 108.1 g.

Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 were obtained from

Sigma Chemical Co. (St. Louis, MO, USA). All other materials were commercial products of the highest grade available.

## 2.2. CK and KM Diet

The CK diet comprised the CE-2 basic rodent diet (Nihon CLEA, Tokyo, Japan) containing 0.25% (w/w) CK. The KM diet comprised the CE-2 basic rodent diet (Nihon CLEA) containing 0.5% (w/w) KM powder.

## 2.3. Animal Experiments

Four-week-old male kwl ICR mice (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan) were housed at  $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and provided with water and the CE-2 diet (Nihon CLEA, Tokyo, Japan) ad libitum. The effects of CK and KM were evaluated by providing the mice with water and the CE-2 diet (Nihon CLEA, Tokyo, Japan) with or without 0.25% (w/w) CK and 0.5% (w/w) KM four weeks ad libitum. The mice were then subcutaneously injected with 0.015 mg/kg of LPS in saline, a concentration that was considerably lower than that applied in the LPS-induced disseminated intravascular coagulation (DIC) model [24]. Blood specimens collected at indicated times from the inferior vena cava using a plastic syringe and needle under pentobarbital (40 mg/kg i.p.) anesthesia later were mixed with 0.2 volumes of 3.2% sodium citrate. Platelet-poor plasma was separated from blood specimens by centrifugation at  $3800 \times g$  for 10 min at  $4^{\circ}\text{C}$  in an MX-100 micro-centrifuge (TOMY, Tokyo, Japan) and stored at  $-80^{\circ}\text{C}$ . All animal experiments proceeded in accordance with the Guide for the Care and Use of Laboratory Animals at Teikyo University and were approved by the Animal Care and Use Committee at Teikyo University (Permission No. 12-013).

## 2.4. Measurement of Plasma PAI-1 Levels and Activity in Mice Plasma

Levels of total PAI-1 and of active PAI-1 antigen (PAI-1 activity) in mouse plasma were measured using the appropriate ELISA kits (Molecular Innovations Inc., Southfield, MI, USA) according to the manufacturer's instructions.

## 2.5. Cell Culture

EA.hy926 cells (ATCC, Manassas, VA, USA) were seeded in Gibco Dulbecco's Modified Eagle's Medium (DMEM basic (1 $\times$ ); Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS) at a density of  $8.0 \times 10^4$  cells per gelatin-coated well in 96-well plates at  $37^{\circ}\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . KM-E dissolved in dimethyl sulfoxide (DMSO) and CK were added to DMEM containing 1% FBS. The final concentrations of KM-E were 10 $\mu\text{g}/\text{mL}$  and 100 $\mu\text{g}/\text{mL}$ . The CK was diluted to 1/100 and 1/1000 in DMEM. The cells were incubated with KM-E or CK for 3 h followed by TNF- $\alpha$  (10 ng/mL) for 24 h. Thereafter, PAI-1 concentrations were measured using total PAI-1 ELISA kits (Molecular Innovations). Cytotoxic ef-

fects of test compounds on EA.hy926 cells were detected using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

## 2.6. Statistics

Data are expressed as means  $\pm$  SD and statistical significance was determined using one-way ANOVA. Data between two groups were compared using Dunnett multiple comparison tests.  $P < 0.05$  was considered to represent significance.

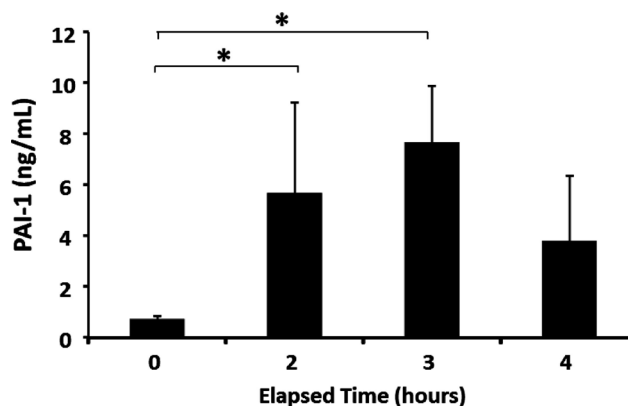
## 3. Results

### 3.1. Effects of LPS on Plasma PAI-1 Levels

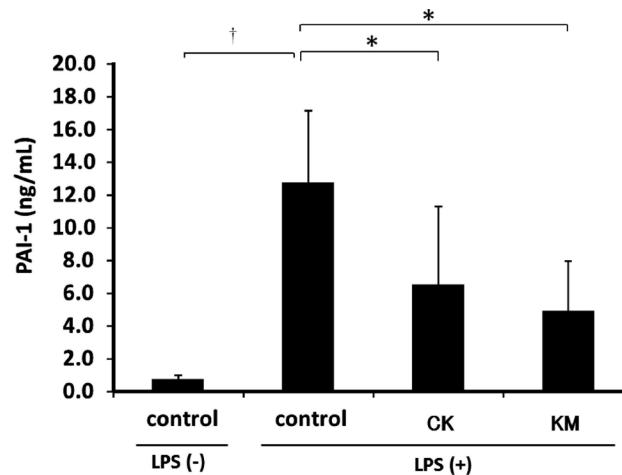
Mice were subcutaneously injected with LPS (0.015 mg/kg), blood was collected at the indicated times, and then total and time-dependent PAI-I antigen levels in plasma was determined. **Figure 1** shows that LPS caused a significant increase in plasma PAI-1 levels that peaked three hours after injection.

### 3.2. The Effects of Oral CK and KM on LPS-Induced PAI-1 Production in Mouse Plasma

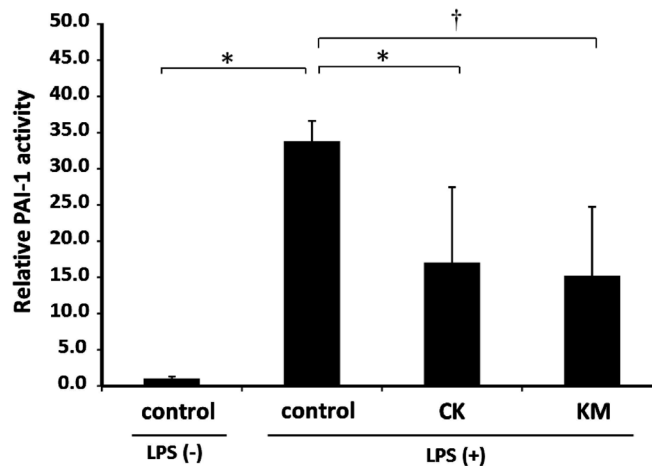
The effects of dietary on LPS-CK and KM induced PAI-1 production in mouse plasma were assessed as follows. The mice were fed with diets containing CK and KM for four weeks and then subcutaneously injected with 0.015 mg/kg of LPS in saline and sacrificed three hours later. **Figure 2** shows that plasma levels of PAI-1 antigen were significantly increased ( $p < 0.001$ ) in mice ( $n = 6$  per group) after being injected with LPS (LPS(+)) compared with control mice that were not injected with LPS (LPS(-)). Orally administered CK and KM significantly suppressed the LPS-induced increase of PAI-1 antigen in mouse plasma (LPS(+) control vs. LPS(+) CK, and LPS(+) control vs. LPS(+) KM;  $n = 6$  mice per group;  $p < 0.01$  for both CK and KM). **Figure 3** shows that LPS increased the level of PAI-1 activity in plasma. Dietary CK and KM for four weeks each



**Figure 1.** Time course of PAI-1 antigen in plasma after LPS injection. Mice were subcutaneously injected with 0.015 mg/kg LPS, blood was collected at indicated times and then total PAI-1 antigen in plasma was determined using ELISA. Data are expressed as means  $\pm$  SD. (0, 2, 4 hours,  $n = 5$ ; 3 hours,  $n = 6$ ). \* $p < 0.05$ .



**Figure 2.** Effects of dietary CK and KM on LPS-induced increase in PAI-1 antigen in mouse plasma. Mice were fed with dietary CK (0.25% w/w) and KM (0.5% w/w) for four weeks. Mice were then subcutaneously injected with LPS (0.015 mg/kg) in saline and sacrificed three hours later. Total PAI-1 antigen in plasma was determined using ELISA. Data are expressed as means ± SD. (n = 6). \*p < 0.01, †p < 0.001.

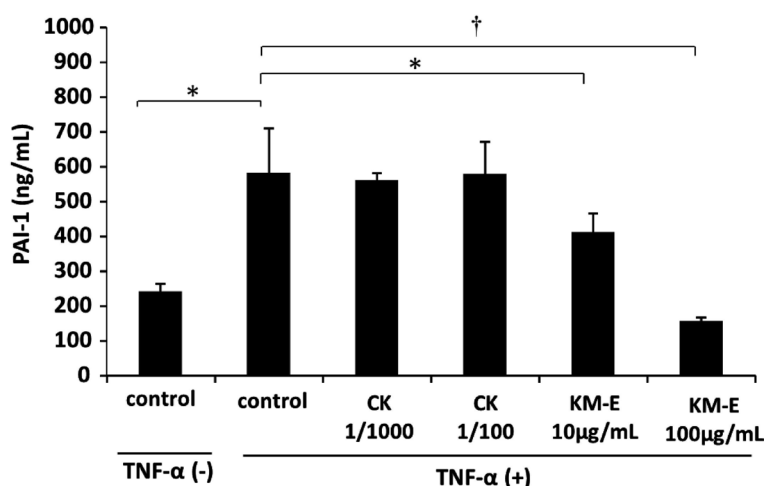


**Figure 3.** Effects of CK and KM on LPS-induced increase in PAI-1 activity in mouse plasma. Mice were fed with dietary CK (0.25% w/w) and KM (0.5% w/w) for four weeks. Mice were subcutaneously injected with LPS (0.015 mg/kg) in saline and sacrificed three hours later. Active PAI-1 antigen levels in plasma were determined using ELISA that detects active PAI-1. Plasma PAI-1 activity is expressed as relative activity of control mice. Data are expressed as means ± SD (control, n = 4; others, n = 5). \*p < 0.01, †p < 0.005.

significantly decreased the LPS-induced increase in PAI-1 activity (LPS(+) control vs. LPS(+) CK, n = 5 mice per group; p < 0.01; (LPS(+) control vs. LPS(+) KM, n = 5 mice per group; p < 0.005).

### 3.3. Effects of CK and KM-E on TNF- $\alpha$ -Induced Increase of PAI-1 in Culture Medium from Endothelial Cell-Like EA.hy926 Cells

**Figure 4** shows the effects of CK and KM-E on TNF- $\alpha$ -induced increase of PAI-1 in culture medium from endothelial cell-like EA.hy926 cells. PAI-1 production was increased in EA.hy926 cells by TNF $\alpha$  stimulation (TNF- $\alpha$ (-) vs. TNF- $\alpha$ (+), p



**Figure 4.** Effects of CK and KM-E on TNF- $\alpha$ -induced PAI-1 increases in EA.hy926 cells. Cells were incubated with CK and KM-E for 3 hours followed by TNF- $\alpha$  (10 ng/mL) for 24 hours. Concentrations of PAI-1 were then measured using total human PAI-1 ELISA. Data are expressed as means  $\pm$  SD. (n = 3). \*p < 0.05, †p < 0.001.

< 0.05; n = 3 per group). Kurozu-moromi ethanol extract dose-dependently suppressed the TNF- $\alpha$ -induced PAI-1 increase in the culture medium (TNF- $\alpha$  (+) control vs. TNF- $\alpha$  (+) KM-E (10 and 100  $\mu$ g/mL), p < 0.05 and p < 0.001, respectively; n = 3 per group). However, CK did not inhibit the TNF- $\alpha$ -induced increase in PAI-1.

#### 4. Discussion

The present findings showed that orally administered CK and KM both significantly suppressed not only LPS-induced increases of PAI-1 antigen but also PAI-1 activity in mouse plasma. This indicated that orally administered CK or KM can decrease thrombotic tendencies induced by inflammation in mice. We found that PAI-1 production was significantly suppressed in the plasma of mice after consuming CK and KM for four weeks, a time-frame that is appropriate to explore anti-thrombotic effects in mice [11] [25] [26].

It is believed that vinegar has the property which improves blood fluidity. Jing *et al* reported that antithrombotic property of grain vinegar is owing to the effect of acetic acid in vinegar [27]. Kurozu and conventional rice vinegars are produced using different raw materials; Kurozu is derived from unpolished rice with rice bran, whereas conventional rice vinegar is derived from polished rice. The brewing processes of Kurozu and conventional rice vinegars are distinctly different [28]. Kurozu contain more abundant amino acids, vitamins organic acids and other compounds than conventional rice vinegar [14] [15] [16]. The concentrated Kurozu (CK) investigated herein was prepared by repeated lyophilization, which means that it was almost completely devoid of acetic acid. Therefore, acetic acid is not involved in the ability of CK to inhibit PAI-1 production. The unpolished rice from which Kurozu is produced contains rice bran and rice germ. Phenolic compounds in rice bran such as ferulic acid and its de-

rivatives including dihydroferulic acid and dihydrosynaptic acid, have various biological properties including anti-inflammatory activity [29]. Then, these phenolic compounds are candidate compounds in CK that can suppress PAI-1. Kurozu-moromi is the sediment left in the bottom of vessels during Kurozu production. Although details remain to be elucidated, Kurozu-moromi contains mainly insoluble substances from unpolished rice including lipids, as well as phenolic compounds and their derivatives. These substances are candidate active compounds in CM that can suppress PAI-1.

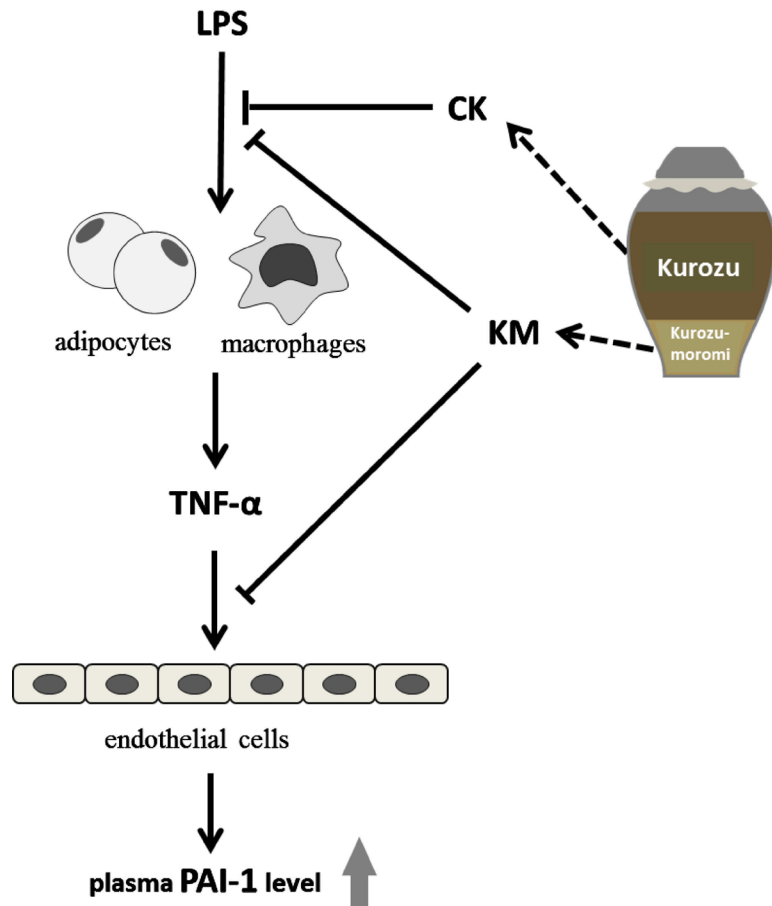
Plasminogen activator inhibitor-1 is expressed in most tissues such as liver, adipose tissues, heart, muscle, bone hematopoietic cells and platelets [30]. Studies of humans and mice have indicated that the liver is one of the main tissues that produce PAI-1 [1]. Adipose tissue-derived PAI-1 is also abundant and is considered important for pathological states such as diabetes, cardiovascular disease, cancer, inflammation [31] [32]. Injected LPS increases PAI-1 mRNA and antigen in adipose tissues [33] [34]. Although the tissue sources of plasma PAI-1 remain controversial, many tissues might be involved. Which tissues are affected by KM and CK remain unclear. However, orally administered dietary KM and CK both significantly suppressed PAI-1 increases induced by LPS in plasma.

Various cytokines influence thrombogenesis, such as TNF- $\alpha$  and interleukin (IL)-6, which are primarily secreted by activated monocytes and macrophages [35]. These cytokines subsequently stimulate vascular endothelial cells to enter a prothrombotic state by expressing prothrombotic factors such as PAI-1 and tissue factor (TF) [35]. The present study also investigated the effects of CK and KM-E on TNF- $\alpha$  induced PAI-1 production in the culture medium of endothelial cell-like EA.hy926 cells. Kurozu-moromi ethanol extract decreased PAI-1 production in the culture medium of these cells, whereas CK did not. However, orally administered CK and KM both significantly suppressed the LPS-induced PAI-1 increase in mice. The reasons for the discrepancy between the effects of CK and KM on TNF- $\alpha$  induced PAI-1 production in EA.hy926 cells and in mice were not clear. It is reported that LPS stimulates TNF- $\alpha$  production from adipocytes and macrophages and produced TNF- $\alpha$  induces PAI-1 expression in endothelial cells [36] [37]. Concentrated Kurozu may decrease plasma PAI-1 level (or PAI-1 production) through only inhibiting TNF- $\alpha$  production of adipocytes and macrophages stimulated by LPS. In contrast, KM may suppress both pathways of LPS-stimulated TNF- $\alpha$  production of adipocytes and macrophages, and TNF- $\alpha$  induced PAI-1 production by directly targeting endothelial cells (Figure 5). Further studies are needed to determine details of the mechanisms by which CM and KM can regulate PAI-1 production.

## 5. Conclusion

Diets containing CK and KM suppressed LPS-induced increase in plasma PAI-1 antigen and activity in model mice. The present findings also showed that KM-E





**Figure 5.** Schematic summary of the effects of CK and KM-E on PAI-1 production.

suppressed TNF- $\alpha$  induced PAI-1 production in the culture medium of EA.hy926 cells. Therefore, Kurozu and Kurozu-moromi might contain some natural products that could decrease thrombotic tendencies in inflammation and thus have potential as antithrombotic foodstuffs.

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### Declaration of Interest

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