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Angiogenic effects of high molecular weight fucoidan in a mouse ischemic limb model

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Abstract:

Background: The biological actions of fucoidan depend on its molecular weight. 20-30 kDa fucoidan has been reported to stimulate angiogenesis in ischemic limbs. We purified very high molecular weight fucoidan (HMWF) from mozuku (brown algae of the Okinawan coontail family) to assess its effect on angiogenesis. **Methods and Results:** We examined the angiogenic effects of mozuku HMWF (300 kDa) and akamoku (Sargassum seaweed) HMWF (80 kDa) in a mouse ischemic limb model by measuring laser Doppler blood flow (LDBF) and capillary density. We also studied the angiogenic actions of mozuku HMWF administered pre- and post-ischemia as compared to post-ischemia treatment. Mozuku HMWF increased both LDBF and capillary density in the ischemic leg, whereas akamoku HMWF did not. Treatment with mozuku HMWF pre- and post-ischemia increased both LDBF and capillary density, which was not seen in post-ischemia treatment alone. **Conclusions:** This study demonstrated the therapeutic effect of pre- and post-ischemia treatment with mozuku HMWF in ischemic limbs, and the timing of administration is important for its angiogenic activity.

Key words:

HMWF, LMWF, Angiogenesis, PAD

Introduction

Peripheral artery diseases (PAD), including arteriosclerosis obliterans (ASO) and Buerger's disease, are characterized by high risk of cardiovascular event, impaired physical performance, and reduced quality of life[1)]. The pathophysiologies of PADs are complex, and are associated with hemodynamic dysfunction, muscular metabolic disorder, inflammation, and oxidative stress[2)]. Although lipid-lowering drugs, anticoagulants, and lifestyle interventions are recommended as standard therapies for PADs[3)], medical treatments for PADs do not sufficiently improve clinical symptoms or long-term outcomes such as cardiovascular related mortality. Thus, new treatment options for patients with PADs are highly desirable.

Fucoidan, which is used in health products[4)], is a complex sulfated polysaccharide found in the cell walls of several types of edible brown algae such as Okinawan mozuku

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(*Cladosiphon okamuranus*) and kombu (*Sccharina japonica*). Fucoidan structure and composition varies among different brown seaweed species, but consists primarily of L-fucose and sulfate groups, along with small quantities of D-galactose, D-mannose, D-xylose, and uronic acid[5,6]. Fucoidan has recently been shown to have beneficial effects in patients with intestinal diseases or metabolic syndrome[7]. Fucoidans also have anti-tumor[8], antioxidant, anti-inflammatory, antiviral, anticoagulant, and antithrombotic activities.

Fucoidans have been reported to ameliorate symptoms of PAD[9]. Fucoidan enhances fibroblast growth factor (FGF)-2 induced angiogenesis, and promotes endothelial cell proliferation and migration[10]. High molecular weight fucoidan (HMWF) is defined as the one with molecular weight > 10 kDa, while low molecular weight fucoidan (LMWF) is defined as the one having molecular weight < 10 kDa[6]. LMWF promotes FGF-2 induced angiogenesis in human endothelial cells[11]. LMWF is reported to potentiate FGF-2 activity and promote angiogenesis in an *in vivo* rat model of hindlimb ischemia[9]. LMWF reportedly enhances the secretion of VEGFA165, which promotes VEGFR-2 binding to neuropilin-1 (NRP1) on the surface of endothelial cells, enhancing neovascularization[12,13]. LMWF also inhibits inflammatory responses, and enhances neovascularization through the eNOS/NO pathway, ameliorating hindlimb ischemia in type 2 diabetic rats[14]. While several reports have shown the angiogenic activity of LMWF, activity of HMWF has not been investigated in details so far. Previous reports have demonstrated an angiogenic activity of HMWF administration post-ischemia[9,14]. The extent of HMWF administration pre-and/or post-ischemic angiogenic activity is unclear. We recently purified HMWF with an average molecular weight of 300 kDa from the brown seaweed Okinawan mozuku (*Cladosiphon okamuranus*). We also purified another HMWF, from the edible seaweed akamoku (*Sargassum horneri*)[15]. The average molecular weight of akamoku HMWF was 80 kDa. In this study, we examined the angiogenic effects of mozuku HMWF (300 kDa) and akamoku HMWF (80 kDa) in a mouse ischemic limb model. We also studied the angiogenic activity of mozuku HMWF administered before and after ischemia (pre- and post-treatment) compared to after ischemia alone (post-treatment).

Methods

Animal care

C57BL/6J mice (male, 8-10 weeks old) weighing 20-25 g were purchased from CREA Japan (Osaka, Japan). Experimental protocols (18-Y-23) were approved by the Institutional Animal Care and Use Committee of Tottori University School of Medicine. Experimental procedures and analyses were performed in a blinded fashion. In some experiments, Lewis rat (male 8-10 weeks old) weighing 200-250 g were used.

Preparation of a unilateral hind limb ischemia model

Unilateral hind limb ischemia was induced in C57BL/6J mice (males, 8-10 weeks) as previously described[16]. On day 0, after the mice were anesthetized by intraperitoneal injection of xylazine hydrochloride (5 mg/kg; Bayer, Tokyo, Japan) and ketamine hydrochloride (80 mg/kg; Sankyo Pharmaceuticals, Tokyo, Japan), the proximal right external iliac artery was ligated using 8-0 silk (MEAR Medic, Chiba, Japan).

Oral administration of mozuku HMWF or akamoku HMWF

Mice were assigned to one of the four groups: 1) the control group (n=7) received saline from day 0 to day 28 (post-ischemia); 2) the pre- and post-treatment with mozuku group (n=6) were given 80 mg/kg/day mozuku HMWF[4] from day -7 to day -1 (pre-ischemia) and 400 mg/kg/day from day 0 to day 28 (post-ischemia); 3) the pre- and post-treatment with akamoku group (n=3) were given 80 mg/kg/day akamoku HMWF from day-7 to day-1 (pre-ischemia), and 400 mg/kg/day from day 0 to day 28 (post-ischemia); 4) the post-treatment with mozuku group (n=3) were given 400 mg/kg/day mozuku HMWF from day 0 to day 28 post-ischemia. Mozuku HMWF and akamoku HMWF (Marine Products Kimuraya Co., Ltd., Tottori, Japan) were administered by oral gavage.

Laser Doppler blood flow analyses

At 0, 7, 14, 21, and 28 days after surgery, lower limb blood flow was measured using a laser Doppler blood flow (LDBF) analyzer (Permed, Stockholm, Sweden). As shown in **Supplemental Figure 1**, Doppler signal was taken as an index of microvascular perfusion of the muscle area depicted by the cursor. We measured LDBF three times at 1-minute intervals, and average values were considered[16]. The results were expressed as a fraction of blood flow in the untreated limb.

Measurement of capillary density

On day 28, the adductor skeletal muscle was isolated from the ischemic limb, embedded in Tissue-Tek 4593 Optimal Cutting Temperature compound (Sakura Finetek Japan, Tokyo, Japan), and snap-frozen in liquid nitrogen. Cryostat sections (8 μm thick) of each specimen were prepared. Sections were incubated with antibodies specific to CD31 (BD Biosciences, San Diego, CA), which were labeled with biotin and detected by avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 2 hours. Labeled cells were defined as vascular endothelial cells. Capillary density was expressed as the number of CD31-positive features per skeletal muscle fiber. Three randomly selected fields from transverse sections were analyzed per animal[16].

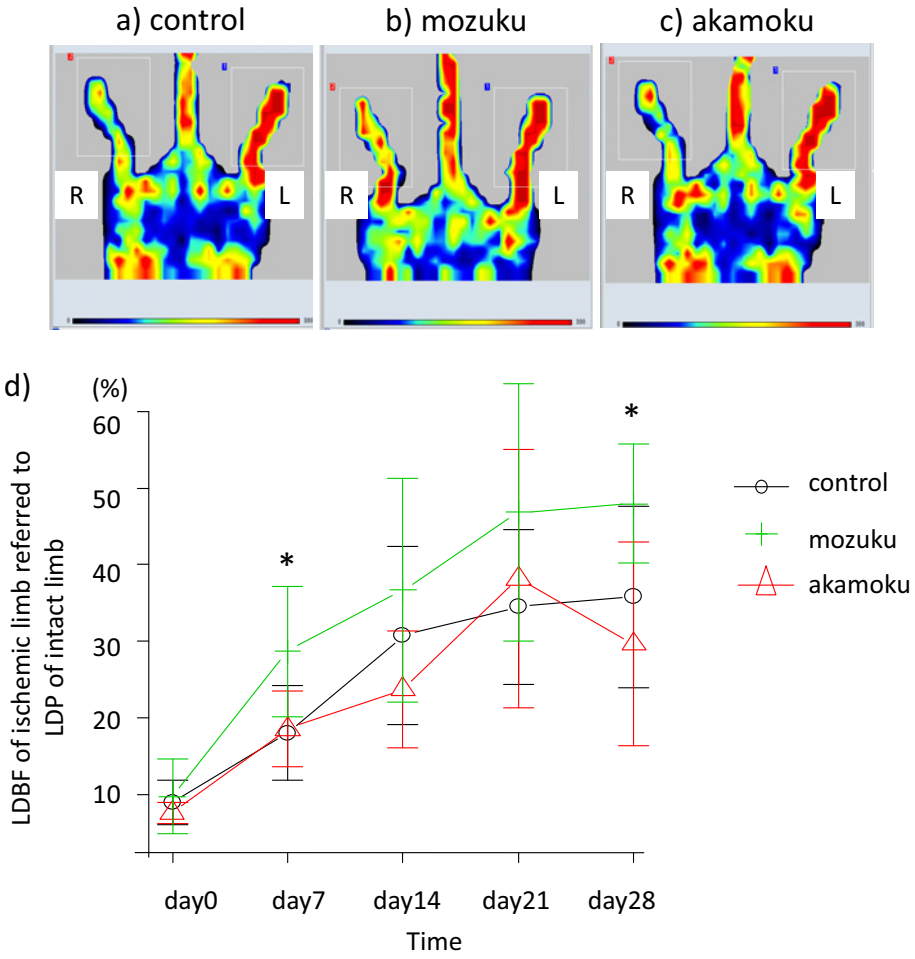

Figure 1. Recovery of blood perfusion in ischemic hind limbs treated pre- and post-ischemia with mozuku HMWF or akamoku HMWF.
Upper panels: Representative images of ischemic lower limbs (right side) of mice in control (a: control), pre- and post-ischemia mozuku HMWF treatment (b: mozuku), and pre- and post-ischemia treatment with akamoku (c: akamoku). Images were obtained on day 28 after ligation of the right external iliac artery. Lower panels: Average time-dependent recovery of blood perfusion in the ischemic hind limbs. Each bar represents mean ± S.D.
control group: administered saline (n=7)
mozuku group: treated pre- and post-ischemia with mozuku HMWF (n=6)
akamoku group: treated pre- and post-ischemia with akamoku HMWF (n=3)
* $P < 0.05$ vs. control group and akamoku group

Chemicals

Fucoidan from Okinawan mozuku (*Cladosiphon okamuranus*) was provided by Marine Products Kimuraya Co., Ltd. (Tottori, Japan). Size exclusion chromatography estimated the average molecular weight of this fucoidan to be 300 kDa. Fucose content was approximately 36% (w/w), and sulfate groups comprised 14% (w/w) of total fucoidan. Moisture content in the powder was 12.8% (w/w). Akamoku HMWF was extracted using the same manufacturing process, at the same facility at Marine Products Kimuraya Co., Ltd. Its average molecular weight was 80 kDa. Its fucose content was approximately 15% (w/w), and sulfate groups comprised 10% (w/w) of total fucoidan. The powder's moisture content was 8.9% (w/w).

Statistical analysis

Data were processed using StatView 5.0 (SAS Inc., NC, USA). All values are expressed as means ± standard deviation (S.D.). Group comparisons were analyzed by one-way ANOVA with post hoc test for multiple comparisons. Perfusion recovery among groups was assessed by repeated-measures ANOVA. Student's t-test was used for two group comparisons. $P<0.05$ was considered as significant.

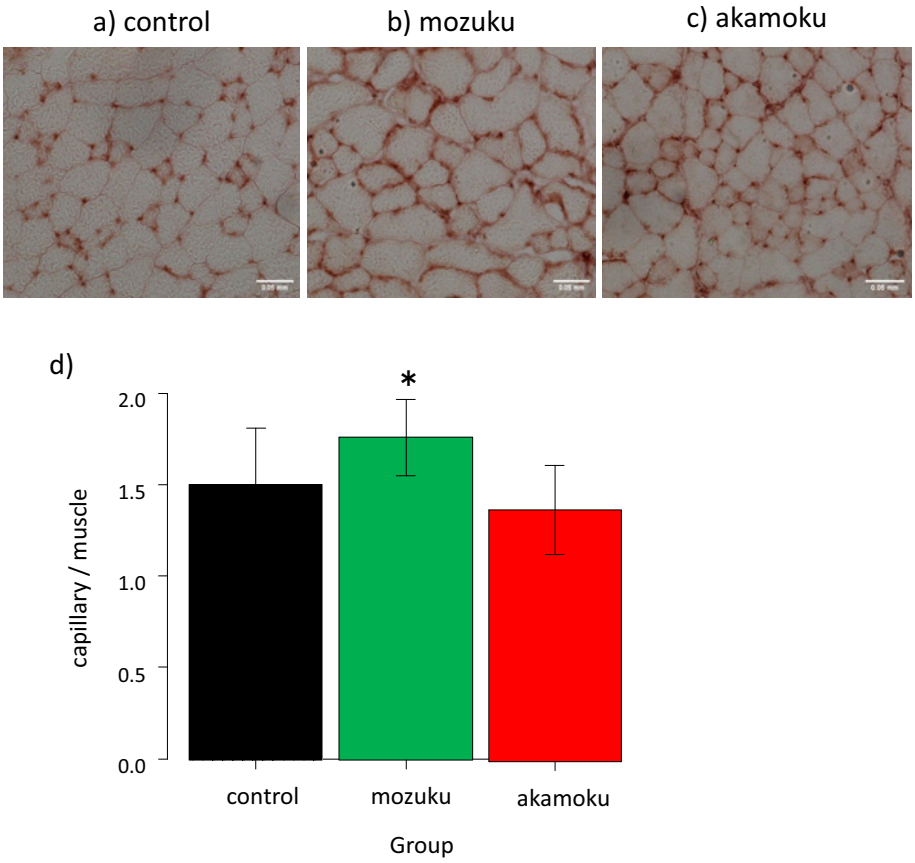

Figure 2. Angiogenic effects of mozuku HMWF and akamoku HMWF compared with control.

Data were obtained 28 days after ligation of the right external iliac artery.

Upper panels: Representative optical micrographs of capillaries in the musculature of the ischemic limb, treated with (a) control, (b) mozuku HMWF (mozuku), or (c) akamoku HMWF (akamoku).

Lower panels: Averaged capillary densities as a ratio relative to skeletal muscle density in ischemic limbs treated with saline (control), mozuku HMWF (mozuku), or akamoku HMWF (akamoku).

* $P < 0.05$ vs. control group and akamoku group

Results

1. Effects of pre- and post-ischemia treatment with mozuku HMWF or akamoku HMWF on blood flow in the ischemic limb

To compare the angiogenic effect of mozuku HMWF with that of akamoku HMWF, we analyzed LDBF in the control group (n=7), the pre- and post-ischemia mozuku HMWF treatment group (n=6), and the pre- and post-ischemia akamoku HMWF treatment group (n=3). **Figure 1** shows representative LDBF in the ischemic limb (right side) in the control group (panel a), mozuku group (panel b), and akamoku group (panel c) at day 28. LDBF in the mozuku group was higher than that in the control or akamoku groups. Panel d shows the time-dependent recovery of blood flow post-surgery from day 0 to day 28 in LDBF experiments. Perfusion recovery was significantly higher in the mozuku group on days 7 and 28 post-ischemia than in the other two groups. The akamoku group showed no improvement throughout this period.

2. Effects of pre- and post-ischemia treatment with mozuku HMWF or akamoku HMWF on capillary density in ischemic limbs

Figure 2 shows the capillaries of the ischemic limb muscle to be immunostained with anti-CD31 antibody at day 28 (control group: panel a, mozuku group: panel b, akamoku group: panel c). Capillary density was increased in the mozuku group compared to that in the control group. It was not found to be increased in the akamoku group. Data obtained from multiple experiments (panel d) showed that capillary density was significantly higher in the mozuku group (1.76 ± 0.07) than in control (1.51 ± 0.10) or akamoku groups (1.38 ± 0.08) ($P<0.05$).

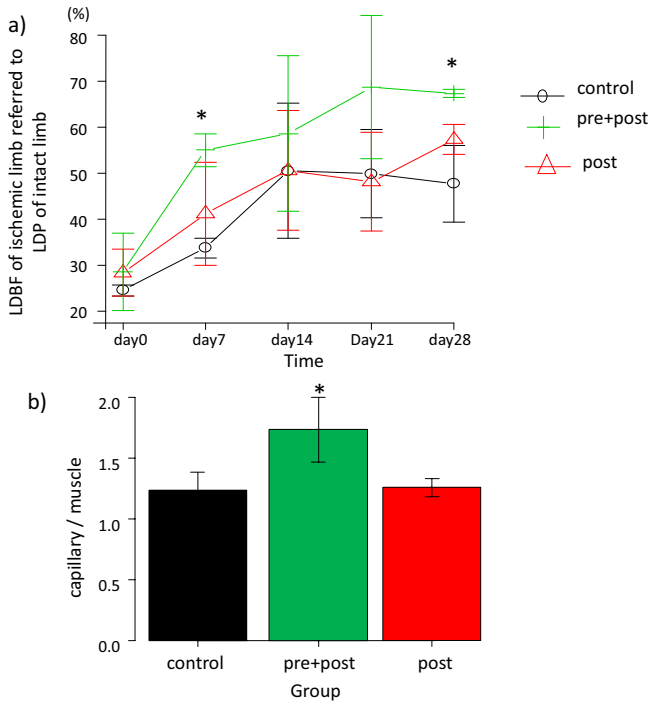

Figure 3. Angiogenic effects of pre- and post-ischemia mozuku HMWF treatment in ischemic limbs, vs. post-ischemia HMWF treatment alone.
Upper panels: Averaged time-dependent recovery of blood perfusion in ischemic hind limbs. Images were obtained 28 days after ligation of the right external iliac artery.
control group: treatment with saline (n=3)
post-ischemia treatment group: post-ischemia mozuku HMWF treatment (n=3)
pre- and post-ischemia treatment group: pre- and post-ischemia mozuku HMWF treatment (n=3)
* P < 0.05 vs. control and post-ischemia treatment groups
Lower panels: Averaged capillary densities relative to skeletal muscle density in control, pre- and post-ischemia treatment, and post-ischemia only treatment groups.
* P < 0.05 vs. control group and post-ischemia treatment group

3. Comparison of effects of pre- and post-ischemia mozuku HMWF treatment on blood flow and capillary density in ischemic limbs to those in limbs treated post-ischemia only

We compared LDBF and capillary density between the control group (n=3), the pre- and post-ischemia mozuku HMWF treatment group (n=3), and the post-ischemia mozuku HMWF treatment group (n=3), as shown in **Figure 3**. In panel a, perfusion of the ischemic limb was significantly increased in the pre- and post-ischemia mozuku HMWF treatment group on days 7 and 28 compared to the control and post-ischemia mozuku HMWF treatment groups.

Pre- and post-ischemia treatment with mozuku HMWF increased the capillary density in the ischemic limb compared to the other two groups (panel b).

Discussion

In the present study, we examined the effects of mozuku and akamoku HMWF on angiogenesis in ischemic limbs, and found that 1) mozuku HMWF improved LDBF and capillary density in ischemic limbs, whereas akamoku HMWF did not show same effects, 2) pre- and post-ischemia mozuku HMWF treatment improved both LDBF and capillary density in ischemic limbs, whereas post-ischemia treatment alone did not improve these two.

Fucoidan consists primarily of L-fucose and sulfate groups, with small quantities of D-galactose, D-mannose, D-xylose, and uronic acid[5]. Fucoidan has shown anti-angiogenic and pro-angiogenic activities depending on its molecular weight[17,18]. In general, HMWF is defined as fucoidan with average molecular weight > 10 kDa. Nakazato et al. examined the effects of oral administration of crude (28 kDa) and purified fucoidan from *Cladosiphon okamuranus* (41.4 kDa) in a rat model[19]. They suggested that both preparations inhibited liver fibrosis by suppressing expression of transforming growth factor-beta (TGF beta), stromal-derived factor 1 (SDF-1), and SDF-1 receptor CXCR[4,19]. Shimizu et al. reported that HMWF, but not LMWF, enhanced innate immunity by decreasing the CD4/CD8 ratio, and increasing the number of cells expressing CD11b[20].

Azofeifa et al. found that HMWF, but not LMWF, showed a therapeutic effect against snake venom *in vivo*, suggesting that HMWF possesses useful biological activities compared to LMWF[17].

LMWF reportedly promotes FGF-2-induced vascular tube formation[11]. By contrast, HMWF with an average molecular weight of 47.5 kDa inhibited angiogenesis[21]. Matsubara et al. showed that HMWF (30 kDa) inhibited tube formation by HUVECs in an *ex vivo* model. In other experiments, HMWF (15-20 kDa) showed a proangiogenic effect. These researchers concluded that HMWF with an average molecular weight of 20-30 kDa would be critical for promoting angiogenesis[18].

In the present study, we used HMWF derived from Okinawan mozuku (300 kDa) and from akamoku (80 kDa). Akamoku HMWF was extracted from *Sargassum horneri*. Several reports have documented potential effects of akamoku HMWF, including anti-inflammatory, antiviral, antioxidant, and antitumor effects[15]. HMWF derived from Okinawan mozuku and akamoku both contain a sulfate group known to bind to VEGF, FGF, and PDGF, thereby promoting an angiogenesis[12,18]. We found that mozuku HMWF (300 kDa) promoted angiogenesis in an ischemic limb model, whereas akamoku HMWF (80 kDa) did not. We confirmed the angiogenic effects of HMWF in a rat model (supplemental Figure 1), confirming the reproducibility of the present data.

Uptake and distribution of fucoidan after oral administration have not been thoroughly studied. However, a growing number of evidences support absorption of fucoidan. Immunodetection of a small amount of fucoidan in human serum

after oral administration has been reported[22]. Tokita et al.[23] reported detection of orally ingested fucoidan in rat serum. These results suggest that orally ingested fucoidan could be broadly absorbed across the mammalian species.

Fucoidan consists primarily of L-fucose and sulfate groups, interspersed with small quantities of D-galactose, D-mannose, D-xylose, and uronic acid[5], and D-galactose[24], D-mannose[25], D-xylose[26], and uronic acid[27] have each been reported to inhibit angiogenic actions, suggesting that either the L-fucose or the sulfate groups in HMWF may play a pivotal role in its angiogenic actions. Further experiments are necessary to clarify the underlying mechanism.

In the present study, pre- and post-ischemia treatment with mozuku HMWF improved the blood flow in ischemic limbs, whereas post-ischemia treatment alone with mozuku HMWF did not. Although these studies imply potential value of using mozuku HMWF as a food supplement for PAD prophylaxis, further clinical studies are needed to support this application.

Our study has some limitations. First, we analyzed LDBF and capillary density in ischemic limbs, but did not investigate mechanisms. Second, we did not study dose-dependency of the effects of pre- and post-ischemia treatment with mozuku HMWF. The dose for post-ischemia mozuku HMWF treatment was 400 mg/kg, five times larger than the pre-ischemia dose. The lack of therapeutic effect of post-ischemia mozuku HMWF treatment alone may be due to the high dose used.

Conclusions

This study demonstrated that pre- and post-ischemia mozuku HMWF treatment had a therapeutic effect in ischemic limbs, and that the timing of mozuku HMWF administration is important for angiogenic activity. Mozuku HMWF may be prophylactically effective against PAD as a health food or supplement.

Conflicts of Interest

Dr. I. Hisatome reported receiving lecturer's fees from Sanwa Kagaku Kenkyusho Co. Ltd, Feizer Co. Ltd. and Fuji Yakuhin Co. Ltd., and research grants from Marine Products Kimuraya Co., Ltd., Teijin Pharma, Fuji Yakuhin Co. Ltd, and Sanwa Kagaku Kenkyusho Co. Ltd.

Ethics approval

The proposed studies were approved by the Institutional Animal Care and Use Committee of Tottori University School of Medicine (18-Y-23).

Abbreviations

HMWF: high-molecular-weight fucoidan
LMWF: low-molecular-weight fucoidan
Da: Dalton
LDBF: laser doppler blood flow

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