Original Paper

Effects of Oligomeric Procyanidins (OPCs) on Human Erythrocyte Filterability and Microcirculation

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Oligomeric procyanidins (OPCs) extracted from pine bark are effective for treating cases of impaired microcirculation because of their antioxidative, antiplatelet and anti-inflammatory activities. For 10 volunteers with mild life style-related diseases, we investigated the rheological effects of OPCs on erythrocyte filterability using a nickel mesh filtration technique and on skin microcirculation using laser Doppler flowmetry. The erythrocyte filterability before the intake of OPCs was lower (64.6 ± 14.5%, P<0.01) than that for the normal controls (85.6 ± 0.8%) and became 69.8 ± 7.7% 1 week after starting intake, and was significantly improved 2 weeks after intake (77.3 ± 5.6%, p<0.02). Correspondingly, laser Doppler flowmeter signals were significantly augmented 1 week (1515 ± 138 mV, p<0.001) and 2 weeks (1570 ± 128 mV, p<0.001) after administration compared with before administration (1335 ± 126 mV). Furthermore, we found a good correlation (R=0.92) between the improvement of filterability and augmentation of the microcirculation. Thus, the present study shows that the combined studies involving the nickel mesh filtration technique and laser Doppler flowmetry are relevant for evaluating the rheological functions of nutritional foods from *in vitro* and *in vivo* standpoints.

Key words: erythrocytes, filterability, microcirculation, laser Doppler flowmetry, oligomeric procyanidins.

1. INTRODUCTION

Oligomeric procyanidins (OPCs) are found in abundance in the bark of the French maritime pine. The bioflavonoid mixture containing OPCs is extracted from the pine bark and commercialized as a water-soluble dry extract. OPCs exert versatile and favorable biological effects such as antioxidative[1, 2], antiplatelet[3] and endothelial protective activities[4]. Therefore, they have been used in Europe for the treatment of impaired microcirculation. Due to the empirical use of OPCs for treating impaired microcirculation, there is little reliable data concerning their effects on human erythrocyte rheology which is closely related to conditions of the microcirculation. Therefore, we investigate the rheological effects of OPCs on human erythrocyte deformability and skin microcirculation, which are impaired by life style-related diseases[5, 6] and smoking[3, 7, 8].

The deformability of erythrocytes that pass through the microvascular network is an essential factor for the maintenance of physiological blood flow in the microcirculation. However, the concept of erythrocyte deformability has no strict definition as a physical quantity, and hence the evaluation of deformability depends on the measurement techniques used and relative sensitivity of the technique to various factors that comprise deformability. Erythrocyte deformability is quantified as erythrocyte filterability (whole cell deformability) using a highly sensitive and quantitative nickel mesh filtration technique[9, 10] which is useful physiologically and clinically[9-14]. However, this technique has not been applied to nutritional or functional food science. Recently, quantitative and reproducible studies have been objectively required in this kind of science, because there have been few such studies performed, and the nickel mesh filtration technique meets such requirements. The filtration technique can be used to assess the physiological bending deformation of intact erythrocytes where deformation *in vivo* involves bending. Therefore, we investigated the physiologic relevance of erythrocyte filterability and compared the results with those of an examination of the *in vivo* microcirculation using laser Doppler flowmetry. This study evaluates the rheological effects of OPCs on erythrocyte filterability and *in vivo* skin microcirculation concerning male subjects whom are affected by several kinds of mild, life style-related diseases.

2. MATERIALS AND METHODS

2. 1 Subjects

The present study was performed according to the Declaration of Helsinki. The study population consisted of 10 Japanese male volunteers including 7 smokers with a mean age of 32.7 ± 6.8 years ranging from 26 to 47 years. They had several episodes of life style-related diseases such as mild obesity, borderline hypertension, mild hyperlipidemia or impaired glucose tolerance. However, they were asymptomatic without histories of medication or hospitalization regarding these disorders. Furthermore, 7 volunteers (4 males and 3 females) without any life style-related diseases served as normal controls with a mean age of 29.9 ± 11.3 years ranging from 19 to 47 years. There was no statistical difference in the mean age between the two groups. Signed informed consent was obtained from each subject prior to commencing the study.

2. 2 Preparation of erythrocyte suspensions

Preparation of the erythrocyte suspensions was performed as described elsewhere[14]. In brief, venous blood from the antecubital vein of the subjects was collected into disposable evacuated syringes with a 21-gauge needle using 1/10 volume of 3.8% trisodium citrate as an anticoagulant. Blood cell counting and hematocrit measurements were carried out using a hemocytometer (Ace Counter, FLC-240A, Fukuda Denshi Co. Ltd., Tokyo, Japan). After centrifugation at 1300 x g for 10 minutes, the plasma and buffy coat were carefully removed and replaced with N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES) sodium salt (HEPES-Na)-buffered saline solution (HBS: 141mM of NaCl, the 10mM of HEPES-Na). The osmolality and pH of the HBS were 287mOsm/kg · H_2O and 7.4, respectively. The osmolality of the HBS was measured using a freezing point depression type osmometer (Fiske Mark 3 Osmometer, Fiske Associates, MA, USA). The erythrocyte suspension was then washed three times by repeated resuspension with HBS and centrifugation at 800xg, 600xg and 500xg for 10 minutes, respectively. The hematocrit value of the erythrocyte suspension was adjusted to 3.0% for the filtration experiments. These procedures were performed within 2 hours after blood sampling.

2. 3 Nickel mesh filter

Fig. 1 shows an electron microscopic photograph of a nickel mesh filter that was produced in accordance with our specifications by a photofabrication technique (Dainippon Printing Co. Ltd., Tokyo, Japan). We specified that this filter should have an outer diameter of 13 mm, a diameter in the filtration area of 8 mm, a thickness of 11 μm, a pore diameter of 3-6 μm, and an interpore distance of 35 μm. The vertical and cylindrical pores were regularly distributed across the filter with no pore coincidence, the pore entrances of which exhibited round and smooth transition into the pore inside. The filter used was a nickel mesh with a pore diameter of 3.09 μm (Tsukasa Sokken Co. Ltd., Tokyo, Japan).

2. 4 Erythrocyte filterability and shape

Filtration experiments were performed using a gravity-based nickel mesh filtration apparatus (Model NOBU-II, Tsukasa Sokken Co. Ltd., Tokyo, Japan) as shown in Fig. 2A. In brief, the hydrostatic pressure (P; mmH_2O) drop -time (t; sec) curve was obtained during continuous filtration by gravity using a pressure transducer.

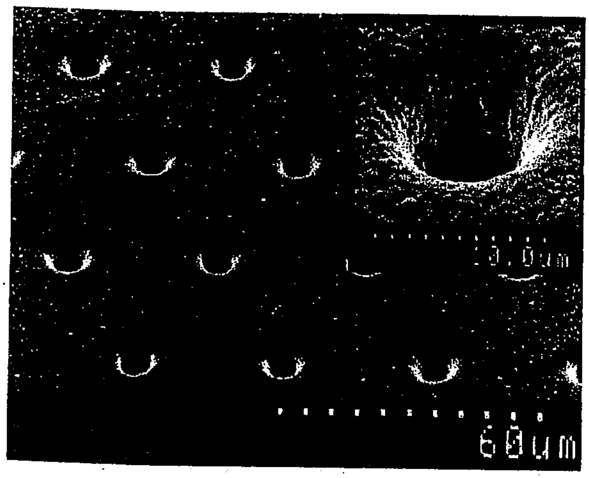

Fig. 1 Scanning electron microscopic photograph of a nickel mesh filter.

Then, P was transformed to a height (h; mm). The tangent of the h-t curve determined by drawing points corresponding to different heights gives the rate of fall of the meniscus in the vertical tube. Thereafter, by multiplying the fall rate by the internal cross-sectional area of the tube, the complete set of flow rates (Q; ml/min) and corresponding P, the P-Q relationship, was calculated[9, 10]. This procedure was automatically performed by measurement software installed on a personal computer. Fig. 2B shows the main window of the measurement software, which contains the operation button, numerical display, graph control, as well as the operation status shown by clicking on the configuration button. Together with the start of the data acquisition process, the measurement software displays the ongoing height and measurement time (h-t) on a graph (Fig. 2B). When the test has been completed, the software displays the pressure and flow rate (P-Q) on a graph (Fig. 2B). The h-t and P-Q graph shown in Fig. 2B was obtained for the filtration of several concentrations of dextran solution. The temperature of the specimens was kept at 25C° by circulating isothermal water through a water jacket surrounding the vertical tube (Fig. 2A). The ratio (%) of the flow rate of the erythrocyte suspension to that of the suspending medium at 100 mmH_2O was used as an index of erythrocyte filterability using the P-Q curve.

An aliquot of the erythrocyte suspension was fixed with isotonic 1.0% glutaraldehyde solution containing 24.5mM NaCl and 50mM phosphate buffer (pH 7.4). The shape of the erythrocytes was then examined using a differential interference contrast microscope (Diaphoto 300, Nikon Co. Ltd., Tokyo, Japan) at 400x magnification.

2. 5 Evaluation of the microcirculation

Microcirculatory conditions of the skin microcirculation were quantified by laser Doppler flowmetry (PeriScan PIM-II, Perimed Co. Ltd.,

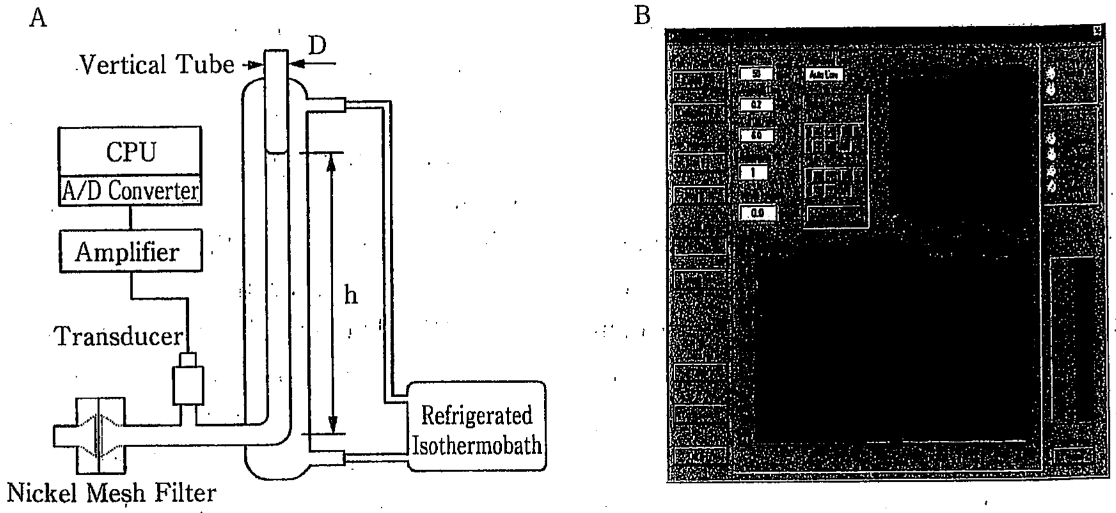

Fig. 2 A nickel mesh filtration system. A: Schematic of the nickel mesh filtration system (see the text). B: Main window of the measurement software (see the text).

Stockholm, Sweden) for which the accuracy and reproducibility of this instrument were previously reported in literature[15-17]. The Doppler shift signal is generally a function of the product of the erythrocytes volume and their mean flow velocity[18]. Thus, laser Doppler flowmetry reflects blood flow in the microcirculation. The Doppler signal was sampled at 10Hz, A/D converted, and then transformed into an output voltage. Autocalibration was intrinsically achieved using this apparatus. The temperature of the laser Doppler scanner facing the skin was standardized to 32C°. After adjusting to room temperature for 20 min, the microcirculation study was started. This instrument noninvasively quantifies the peripheral microcirculation either in a 2-dimensional color-coding mode or in a one-point monitoring mode as a function of time. The present study adopted the former color mode suitable for the planner imaging of superficial microcirculation. The maximum scanning area was 30cm × 30cm and the maximum scanning speed was 90 sec. The mean superficial blood flow was calculated by averaging the 256 × 256 pixels. After taking a 10 min rest, each subject put their right hand on the plate over which the laser Doppler scanner was positioned. The area of interest was not altered among the subjects and during the study period. This examination was performed from 10 to 11 am in a quiet room.

2. 6 Study protocols

OPCs are commercialized as water-soluble fine granules (Flavangenol®, Toyo Shinyaku Co. Ltd., Tosu, Saga 841-0002, Japan). Flavangenol® at 40 mg/day was orally administered to the ten male subjects every day for two weeks. Erythrocyte filterability and the skin microcirculation were evaluated during the control period and 1 and 2 weeks after starting oral administration. Life style including smoking was not altered during the study period. Likewise, erythrocyte filterability for the seven normal controls was also estimated. These experiments were performed at room temperature (22 ± 3C°). The study protocol was approved by the ethical committee of the Institute of Rheological Function of Foods Co. Ltd. (Kubaru, Hisayama, Fukuoka 811-2501, Japan).

2. 7 Data analyses

The data was expressed as means ± SD. Data analyses were performed using the paired or unpaired Student's t-test, and practical computation was performed using Microsoft Excel 2000 on Windows 98/XP (Microsoft, Tokyo, Japan).

Differences with a p value<0.05 were considered to indicate statistical significance.

3. RESULTS

3.1 Oral administration of OPCs

Among the 10 male subjects with several kinds of life style-related diseases, 7 were smokers, whereas the 7 control subjects were non-smokers. OPCs (Flavangenol® at 40 mg/day) were orally administered to the 10 subjects every day for two weeks. During the study, there were no adverse effects caused by the OPCs and none of the subjects was dropped out.

3.2 Representative P-Q relationships under OPCs administration

Fig. 3 shows the representative P-Q relationships for saline and several kinds of the erythrocyte suspension used during the continuous filtration experiments. The filtration system contains a data analysis support program installed on a personal computer, which has an option that allows a graphical display and representative point sampling of the data acquired during the test to be accessed by Microsoft Excel 2000. Thus, we could easily display a P-Q graph such as that shown in Fig. 3. The P-Q relationship for HBS were linear with the line passing through the origin (×), indicating the Newtonian behavior of HBS. The linearity was reproducible when it was filtrated using the nickel mesh filtration method. In contrast, the P-Q relationships for the erythrocyte suspensions displayed smooth curves convex along the abscissa over the low-pressure region, revealing non-Newtonian characteristics of the suspensions. The flow rate for the erythrocyte suspension for a normal control (□) was higher than that for three representative subjects at any given pressure, irrespective of treatment with OPCs. Moreover, the flow rate for the erythrocyte suspension for the three subjects 2 weeks after starting the oral intake of OPCs (◇, △, ○) was higher than that for the subjects over the control period (◆, ▲, ●) at any given pressure, showing that the filterability of erythrocytes after administration was improved compared with that before administration.

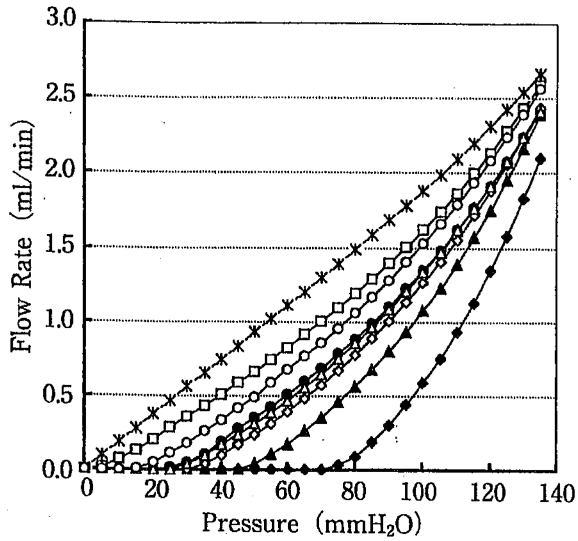

Fig. 3 Representative relationships between the pressure (P; mmH_2O) and flow rate (Q; ml/min) during the continuous filtration experiment. The P-Q relationships show saline (×), normal control (□), and 3 representative subjects before (◆, ▲, ●) and after (◇, △, ○) the oral administration of 40 mg/day OPCs, respectively. The hematocrit value of the erythrocyte suspension was 3%.

3.3 The effects of OPCs on erythrocyte filterability

Figure 4 shows the effects of oral intake of OPCs on erythrocyte filterability. The mean erythrocyte filterability for the 7 normal controls was 85.6 ± 0.8%, which was compatible with our previous finding obtained using a nickel mesh filter with a pore size of 3.20 μm[19]. The erythrocyte filterability for the 10 subjects with life style-related diseases before OPCs administration was 64.6 ± 14.5%, which was statistically lower than that for the controls ($p<0.01$), whereas the filterability for the subjects 1 week after starting OPCs administration was 69.8 ± 7.7%, showing a tendency toward improvement. Furthermore, the filterability for the subjects 2

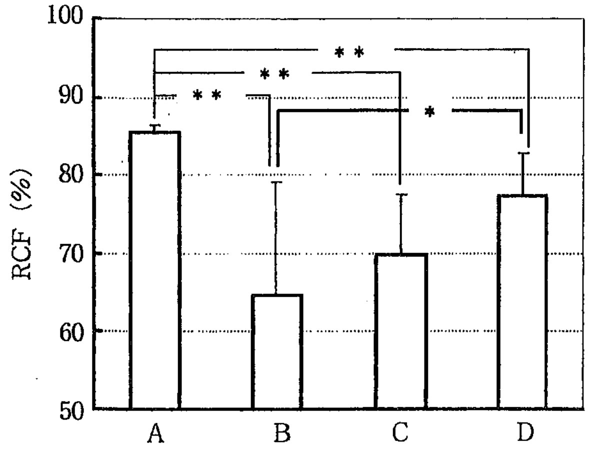

Fig. 4 The effects of the oral administration of OPCs on erythrocyte filterability (%). The bar indicates 7 normal controls (A), 10 male subjects before (B), 1 week (C) and 2 weeks (D) after the oral administration of 40 mg/day OPCs. The data is expressed as means ± SD (*: $P<0.05$, **: $P<0.01$).

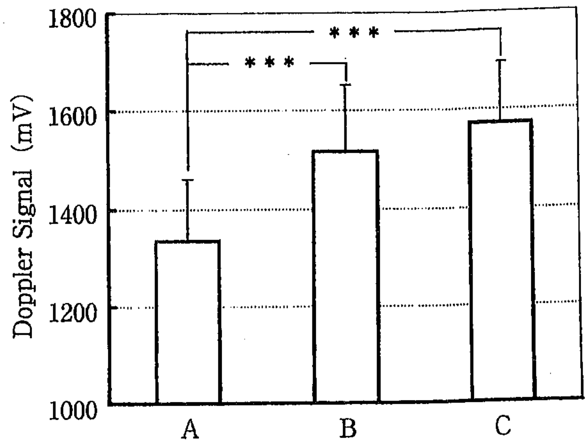

Fig. 5 The effects of the oral administration of 40 mg/day OPCs on the skin microcirculation evaluated by laser Doppler flowmetry. Laser Doppler signals that reflect the extent of the microcirculation were expressed in terms of voltage (mV). The bar indicates male subjects before (A), 1 week (B) and 2 weeks (C) after the oral administration of 40 mg/day OPCs. The data is expressed as means ± SD (***: $P<0.001$).

weeks after starting administration was significantly higher at 77.3 ± 5.6% ($p<0.05$) than that before administration, but was still significantly ($p<0.01$) lower than that for the controls. These results show the apparent improvement of impaired erythrocyte filterability attributable to the oral intake of OPCs at least for the subjects suffering from various kinds of mild life style-related diseases. The mean corpuscular volume (MCV) of 86.1 ± 3.5 μm^3 and the mean corpuscular hemoglobin concentration (MCHC) of 33.8 ± 0.9% of the erythrocytes of the subjects before OPCs administration were about the same as those in normal controls with MCV being 86.9 ± 1.9 μm^3 and MCHC being 34.0 ± 1.1%. Also, the erythrocytes did not show any discernible shape changes (data not shown).

3. 4 The effects of OPCs on skin microcirculation

We investigated the microcirculatory conditions of the skin microcirculation using laser Doppler flowmetry to examine the blood flow in the microcirculation. Flowmetry can be used to express blood flow, the extent of the microcirculation, as a voltage (mV). Fig. 5 shows the effects of the oral intake of OPCs on the microcirculation for the 10 subjects with life style-related diseases. The extent of the microcirculation in the subjects before the intake of OPCs was 1335 ± 126mV, and was markedly augmented to 1515 ± 138mV ($p<0.001$) 1 week after administration and to 1570 ± 128mV ($p<0.001$) 2 weeks after administration. Furthermore, we found a good correlation ($y= 17.81x + 216.6$, $R=0.92$) between the improvement in erythrocyte filterability (x) and the increase in the extent of the microcirculation quantified by the laser Doppler signals (y). This revealed a close relationship between the *in vitro* results, the erythrocyte filterability assessed by the nickel mesh filtration technique, and the *in vivo* results, the skin microcirculation examined by laser Doppler flowmetry.

4. DISCUSSION

Recently, oligomeric procyanidins (OPCs) have attracted considerable public attention as an excellent food source in Japan as in Europe, because many kinds of nutritional or functional foods contain large numbers of procyanidins including OPCs, catechin and epicatechin. Therefore, the study on the rheological effects of OPCs is required for clinical as well as basic science. In particular, such study is essential for nutritional science when nutritional foods are used to prevent life style-related diseases and to establish a healthy life style. The present study based on the nickel mesh filtration technique and laser Doppler flowmetry satisfies these requirements as described below.

The erythrocyte filterability assessed by the nickel mesh filtration technique was impaired in subjects with mild life style-related diseases (Figs. 1 and 2). Erythrocyte filterability is mainly determined by membrane properties, the cellular internal viscosity, which is reflected in the MCHC, and the geometric factors of erythrocytes that reflected in the surface area to volume ratio such as the MCV and changes in shape[9, 10]. In the present study, the MCHC of erythrocytes of the subjects was about the same as that of the normal controls, suggesting that the impaired filterability of the subjects is not attributable to an increase in the cellular internal viscosity. Nickel mesh filters with narrow pores such as 3.09 μ m in diameter as used in this study are especially sensitive to small changes in the MCV of erythrocytes[10]. However, the MCV of the subjects was the same as that of the normal controls and no discernible shape change was observed, suggesting that the impaired filterability was not caused by changes in the geometric factors. These results indicate that impaired filterability mainly arises from changes in the membrane properties. In our previous filtration study using nickel mesh with narrow pores, we noted impaired erythrocyte filterability for subjects with mild hyperlipidemia who were healthy[19]. Various kinds of life style-related diseases including hyperlipidemia are more or less associated with oxidative stress[20, 21], which is thought to cause not only vascular endothelial injury but also the impairment of erythrocyte filterability as shown in our previous study[14, 22].

Passive as well as active smoking generates various kinds of reactive oxygen species (ROS) and causes oxidative stress[23-25]. ROS induces progressive phospholipid peroxidation of the erythrocyte membrane and reduces erythrocyte membrane fluidity[26-30] and erythrocyte filterability[14, 22]. Considering that the impaired filterability of the subjects among whom 7 were smokers was improved by the administration of OPCs that exert potent antioxidative effects[1, 2], the impairment of filterability seems to mainly arise from oxidative stress caused by ROS, and thereby due to changes in the membrane properties. Our recent study showed profound impairment of intact human erythrocyte filterability caused by the organic ROS donor, tert-butyl hydroperoxide (tBHP)[14]; which has been widely used as a model system because it exerts oxidative stress on human erythrocytes[26-30]. The severe impairment of erythrocyte filterability arose from phospholipid peroxidation and protein degradation of the erythrocyte membrane[14]. In addition, verapamil, a representative Ca^{2+}-channel blocker, improved impaired erythrocyte filterability through its mechanism of action via Ca^{2+}-mediated signaling pathways. In general, ROS generation involved in life style-related diseases or smoking is not great compared with tBHP-mediated ROS generation, but the exposure time for ROS for the subjects was considerably long. In light of this oxidative stress, the impairment of erythrocyte filterability in the subjects may have been attributed to the prolonged exposure to ROS, leading to changes in the membrane properties. In addition, the marked improvement in erythrocyte filterability observed for the oral intake of OPCs seems to arise from the potent antioxidative effects of OPCs.

Laser Doppler flowmetry used for the measurement of tissue blood flow in the peripheral microcirculation is a modern, noninvasive technique which does not involve contact with the blood vessel, and newly developed equipment can provide for stability and reproducibility[18]. This technique has been applied not only to examination of the microcirculation of cutaneous but also to that of the intestine[16], nasal mucosa[15], and surface of the brain[17]. The present study found that the significant augmentation of laser Doppler signals can be brought about by the oral intake of OPCs (Fig. 3). Here, erythrocyte filterability plays a role in maintaining physiologically fluent microcirculation. We also found a good correlation between the improvement of erythrocyte filterability and the increase in blood flow in the microcirculation. These findings indicate that the improvement in filterability may be directly linked to fluent microcirculation. To our knowledge, this is the first quantitative study to show the close relationship between erythrocyte filterability and the microcirculation. The good correlation between the improvement of erythrocyte filterability and the augmentation of the microcirculation can be attributed to the assessment of filterability via the nickel mesh filtration technique that permits the evaluation of the physiological, *in vivo* bending deformation of intact erythrocytes. Thus, the combined use of the nickel mesh filtration technique and laser Doppler flowmetry allowed us to evaluate the rheologic functions of nutritional foods in a quantitative and reproducible manner from *in vitro* and *in vivo* standpoints.

CONCLUSIONS

The present combined study with the nickel mesh filtration technique and laser Doppler flowmetry revealed that impaired erythrocyte filterability is closely related to disturbed microcirculation *in vivo*. OPCs play a role in ameliorating erythrocyte filterability and the microcirculation of subjects with life style-related diseases or those who smoke, probably through the antioxidative action of OPCs.

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