Original Paper

Impaired Filterability of Erythrocytes from Subjects with Cold Intolerance

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Female subjects with cold intolerance complain of extreme coldness accompanied with swelling, blanching and numbness of extremities, and many related feelings of discomfort. Some disorders of microcirculation and a remarkable decrease in estrogen followed by menopause have been suspected as pathophysiological causes of cold intolerance. We have investigated the filterability of erythrocytes from premenopausal and postmenopausal subjects with cold intolerance, because disturbed microcirculation *in vivo* is closely related to the impaired erythrocyte filterability. The mean erythrocyte filterabilities for premenopausal subjects with cold intolerance (84.6 ± 2.2%, n=23) and postmenopausal cases (84.8 ± 2.3%, n=24) were significantly ($p<0.001$) lower than the filterability of controls (87.7 ± 1.0%, n=9). However, there was no difference in the filterability between the premenopausal and postmenopausal subjects, suggesting that estrogen levels may not play a major role in the pathophysiology of impaired filterability and cold intolerance. Furthermore, we investigated the rheologic effects of oligomeric procyanidins (OPCs) in these subjects, because OPCs show antioxidative actions and improve microcirculation. However, there were no effects of OPCs on filterability. In conclusion, impaired filterability of erythrocytes from subjects with cold intolerance is not related to oxidative stress or lack of estrogen, but possibly to microvascular structure and function in cold intolerance.

Key words: erythrocytes; filterability; cold intolerance; oligomeric procyanidins

1. INTRODUCTION

There are many females with cold intolerance (so-called "Hie-sho")[1, 2] in Japan. They complain of extreme coldness, blanching, and numbness of fingers and toes, even during summer, which sometimes lead to insomnia at night. Furthermore, they have recurrent episodes of general malaise, fatigability, loss of appetite, irregular menstruation, pedal swelling, and vague aches, thereby becoming restless, irritable, and uncomfortable. Therefore, their quality of life is impaired. Many causes have been considered for the pathophysiology of cold intolerance, such as autonomic nervous system imbalance leading to thermoregulatory dysfunction, a disorder of peripheral microcirculation, a nutritional or metabolic disorder related to impaired thermogenesis[3], and hormonal imbalance, in particular, due to a remarkable decrease in circulating estrogen after menopause. However, there are little reliable scientific data about the pathophysiology of cold intolerance.

The deformability of erythrocytes that pass through the microvascular network is an essential factor for the maintenance of physiological microcirculation. Recently, we clearly showed that disturbed microcirculation *in vivo* is closely related to impaired erythrocyte filterability (whole cell deformability)[4], using laser Doppler flowmetry[5] and a highly sensitive and quantitative nickel mesh filtration technique that we have developed[4, 6, 7]. It is to be noted here that this filtration technique is quite useful to investigate the filterability of erythrocytes of inherited hemoglobin disease[8, 9] or those subjected to oxidative stress[10]. Oxidative stress is reportedly augmented by menopause[11], because estrogen exerting antioxidative actions[12, 13] is reduced profoundly by menopause[14]. Recently, we reported the rheologic benefit of oligomeric procyanidins (OPCs), which are extracted from pine bark and exert antioxidative[15] and antiplatelet[16] activities. OPCs improve the impaired filterability of erythrocytes from smokers and subjects with metabolic syndrome[4], and oxidative stress is closely linked to smoking[17-19] and metabolic syndrome[20]. Antioxidative supplementation is also recommended as an alternative to hormone replacement therapy for menopausic women[11].

Thus, the aim of this study was to investigate the filterability of erythrocytes from subjects with cold intolerance, and the effects of OPCs on the erythrocyte filterability in these subjects, using the nickel mesh filtration technique, and thereby to consider the pathophysiology of cold intolerance from a rheologic viewpoint.

2. MATERIALS AND METHODS

2. 1 Subjects

The present study was performed according to the Declaration of Helsinki. The study population consisted of 24 Japanese postmenopausal female volunteers with a mean age of 59.0 ± 6.4 years, ranging from 52 to 75 years (Subjects 1: S1), and 23 Japanese premenopausal female volunteers with a mean age of 30.7 ± 9.1 years, ranging from 22 to 50 years (Subjects 2: S2). They had several subjective complaints of cold intolerance: 1) swelling of the extremities accompanied with extreme coldness, 2) insomnia due to coldness of the extremities, 3) a need to use gloves and/or socks during the cooler months, even while staying indoors, 4) headache, shoulder stiffness and vague ache, and/or lumbago induced by cooling during summer. However, they did not accept medication or hospitalization with respect to these symptoms. Furthermore, 9 volunteers without cold intolerance (4 males and 5 females) served as normal controls, with a mean age of 28.2 ± 10.5 years ranging from 19 to 47 years, based on our previous study demonstrating no gender difference and no age-dependency in the mean erythrocyte filterability assessed by the same method as in this study[21].

2. 2 Preparation of erythrocyte suspensions

Preparation of the erythrocyte suspensions was performed using a conventional method. In brief, venous blood from the antecubital veins of subjects was collected into disposable evacuated syringes with a 21-gauge needle using a 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 (HEP-

ES-Na)-buffered saline solution (HBS: 141mM of NaCl, 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 each, respectively. The final 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 Erythrocyte filterability and shape

Filtration experiments were performed with a gravity-based filtration apparatus (Model NOBU-II, Tsukasa Sokken Co., Ltd., Tokyo, Japan) using a nickel mesh filter with a pore diameter of 4.55 μm (Tsukasa Sokken Co., Ltd., Tokyo, Japan). In brief, the hydrostatic pressure (P; mmH_2O) drop-time (t; sec) curve was obtained during continuous filtration by gravity using a pressure-voltage transducer. 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 (Fig. 1). This procedure was automatically performed by measurement software installed on a personal computer. Together with the start of the data acquisition process, the measurement software displays the ongoing height and measurement time (h-t) on a graph. When the test is completed, the software displays the pressure and flow rate (P-Q) on a graph[4]. The temperature of the specimens was kept at 25C° by circulating isothermal water through a water jacket surrounding the vertical tube (Fig. 1).

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 interfer-

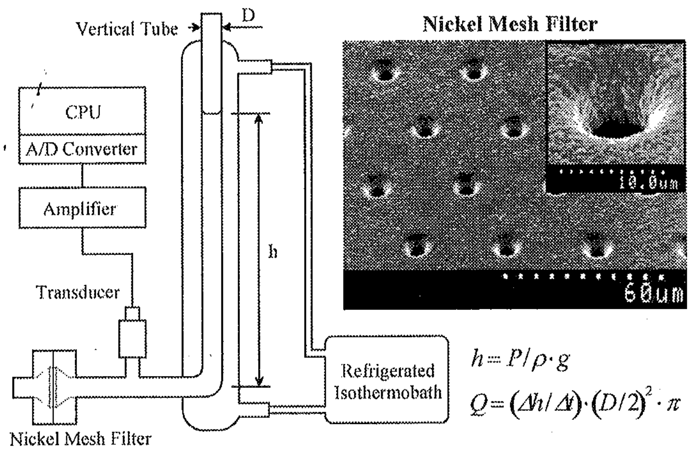

Fig. 1 : Schema of the nickel mesh filtration system (see text) and a scanning electron microscopic photograph of a nickel mesh filter. The filter has an outer diameter of 13 mm, a filtration area diameter of 8 mm, a thickness of about 11 μm, a pore diameter of 3-6 μm, and an interpore distance of 35 μm. The vertical and cylindrical pores are regularly distributed across the filter with no pore coincidence, the pore entrances of which show round and smooth transition into the inside of the pore.

ence contrast microscope (Diaphoto 300, Nikon Co., Ltd., Tokyo, Japan) at 400x magnification.

2. 4 Study protocols

OPCs are commercialized as water-soluble fine granules (Flavangenol®, Toyo Shinyaku Co., Ltd., Tosu, Saga 841-0002, Japan). The recommended dose of Flavangenol® (40 mg/day) was orally administered to the 47 female subjects every day for 2 weeks. Erythrocyte filterability was evaluated during the control period and 2 weeks after starting oral administration. Life style habits were not altered during the study period. 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. 5 Data analyses

The data was expressed as means ± SD. Data analyses were performed using the paired or nonpaired 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 significance.

3. RESULTS

3. 1 Representative P-Q relationships

Fig. 2 shows the representative P-Q relationships for the HBS (saline) and the several kinds of erythrocyte suspension used in the continuous filtration experiments. The P-Q relationship for the HBS was linear with the line passing through the origin (○), indicating the Newtonian behavior of the HBS. In contrast, the P-Q relationships for the erythrocyte suspensions displayed smooth convex curves 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 with cold intolerance (▲, ◆, ■) at any given pressure. The ratio (%) of the flow rate of the erythrocyte suspension to that of the HBS at 100 mmH_2O was used as an index of erythrocyte filterability using the P-Q relationship.

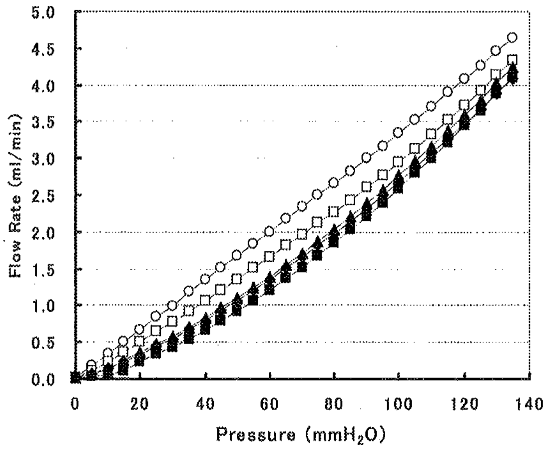

Fig. 2 : Representative relationships between the pressure (P; mmH_2O) and flow rate (Q; ml/min) during the continuous filtration experiment. The P-Q relationships show HBS (○), normal control (□), and 3 representative subjects with cold intolerance (◆, ▲, ■), respectively. The hematocrit value of the erythrocyte suspension was 3%.

3. 2 Oral administration of OPCs

OPCs (Flavangenol® at 40 mg/day) were orally administered to the 47 subjects with cold intolerance every day for 2 weeks. During the study, there were no adverse effects caused by the OPCs and none of the subjects dropped out.

3. 3 Filterability of erythrocytes from subjects with cold intolerance

Fig. 3 shows the filterabilities of erythrocytes from normal controls and premenopausal or postmenopausal subjects with cold intolerance investigated before or after starting the administration of OPCs. The mean erythrocyte filterability for the 9 normal controls was 87.7 ± 1.0%. The erythrocyte filterability for the 24 postmenopausal subjects with cold intolerance (S1)

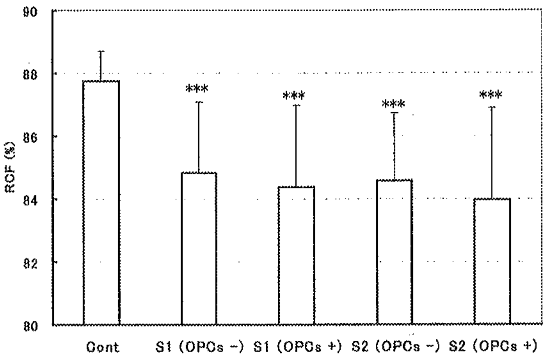

Fig. 3 : Filterability of erythrocytes from subjects with cold intolerance. RCF means erythrocyte (red cell) filterability. The bar indicates 9 normal controls (Cont.), 24 female subjects with cold intolerance after menopause [S1 (OPCs -)], those after the administration of OPCs [S1 (OPCs +)], 23 female subjects with cold intolerance before the menopause [S2 (OPCs -)], and those after the administration of OPCs [S2 (OPCs +)]. Erythrocyte filterability in subjects with cold intolerance was significantly lower than that in controls ($p<0.001$), irrespective of menopause and the administration of OPCs. The data are expressed as means ± SD (***: $P<0.001$).

before administration of OPCs was 84.8 ± 2.3% and that for the subjects after starting administration of OPCs was 84.4 ± 2.6% (Table 1). These values were significantly lower than the filterability of the controls ($p<0.001$), showing the decreased filterability of erythrocytes from these subjects and no effects of OPCs on the filterability. In addition, the erythrocyte filterability for the 23 premenopausal subjects with cold intolerance (S2) prior to the OPCs administration was 84.6 ± 2.2% and that for the subjects after starting the administration was 84.0 ± 2.9% (Table 2). These filterabilities were significantly lower than the fiterability of the controls ($p<0.001$), showing exactly the same tendency as those in S1.

3. 4 Hematological examination

The hemoglobin (Hb) content (12.7 ± 1.3 g/dl) of the 24 postmenopausal subjects with cold intolerance (S1) was lower than that (14.0 ± 1.9 g/dl) of the normal controls ($p<0.05$), showing very slight anemia (Table 1). The Hb content (12.5 ± 1.1 g/dl) and Ht values (37.5 ± 2.4 %) of the 23 premenopausal subjects with cold intolerance (S2) were also lower than those (14.0 ± 1.9 g/dl, 40.9 ± 5.2 %) of the normal controls ($p<0.01$, $p<0.05$, respectively), showing slight anemia (Table 2). Other results of hematological examinations of the subjects with cold intolerance were within normal ranges (Tables 1 & 2). In particular, it is to be noted here that the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) of the erythrocytes of the subjects were about the same as those in normal controls; these hematological parameters are major determinants of erythrocyte filterability[6, 7]. Moreover, the erythrocytes of the subjects did not show any discernible shape changes (data not shown).

4. Discussion

We clearly demonstrated that erythrocyte filterability is impaired in subjects with cold intolerance irrespective of menopause (Figs. 2 & 3, Tables 1 & 2) and that OPCs have no rheologic beneft in these subjects.

Erythrocyte filterability is mainly determined by the membrane properties, the cellular internal viscosity reflected by MCHC, and the cellular geometric factors such as the surface area to volume ratio reflected by MCV and cellular shape changes[6, 7]. In the present study, the MCHC of erythrocytes of the subjects with cold intolerance was about the same as that of the normal controls (Tables 1 & 2), suggesting that the impaired filterability of the subjects is not attributable to an increase in the cellular internal viscosity. Also, the MCV of the subjects was about the same as that of the normal controls (Tables 1 & 2), and no discernible shape changes were observed, suggesting that the impaired filterability is not caused by changes in geometric factors. These results indicate that the impaired filterability mainly arises from changes in the cellular membrane properties.

Concerning the pathophysiology of cold intolerance, microcirculation disorder and the remarkable decrease in estrogen accompanied by menopause are considered to be the major etiologies. Estrogen plays an important role in maintaining physiological circulation through its antioxidative[12,13] and vasodilating[22] actions. However, in the present study, the comparison of erythrocyte filterability in premenopausal and postmenopausal subjects indicates that erythrocyte filterability is impaired age-independently in subjects with cold intolerance and that estrogen showing profound fall in menopause[14] does not play a major role in influencing erythrocyte filterability. This finding is compatible with the previous reports, which claim that cold intolerance is age-independent[1,2].

Table 1

	Cont.	S1	P (Cont.-S1)	S1+OPCs	P (S1-S1+OPCs)
Age (years)	28.2±10.5	59.0±6.4	0.001	-	-
Ht (%)	40.9±5.2	37.9±3.5	N. S.	37.3±3.4	N. S.
Hb (g/dl)	14.0±1.9	12.7±1.3	0.05	12.6±1.4	N. S.
MCV (μm^3)	89.9±2.6	92.4±3.9	N. S.	92.0±3.8	N. S.
MCHC (%)	34.2±0.5	33.6±1.0	N. S.	33.8±1.1	N. S.
Plt. ($10^4/mm^3$)	22.6±4.2	21.8±4.8	N. S.	22.1±4.9	N. S.
WBC ($10^3/mm^3$)	4.4±1.5	4.8±0.8	N. S.	4.7±0.7	N. S.
RCF (%)	87.7±1.0	84.8±2.3	0.001	84.4±2.6	N. S.

S1 = 24 female subjects whose menstruation has ceased; Cont. = normal controls; P (Cont.-S1) = P value between Cont. and S1; S1+OPCs = S1 after the administration of OPCs; P (S1-S1+OPCs) = P value between S1 and S1+OPCs. The data are expressed as means ± SD.
Ht, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell; Plt., platelet; RCF, erythrocyte (red cell) filterability.

Table 2

	Cont.	S2	P (Cont.-S2)	S2+OPCs	P (S2-S2+OPCs)	P (S1-S2)
Age (years)	28.2±10.5	30.7±9.1	N. S.	-	-	0.001
Ht (%)	40.9±5.2	37.5±2.4	0.05	37.3±2.7	N. S.	N. S.
Hb (g/dl)	14.0±1.9	12.5±1.1	0.01	12.4±1.1	N. S.	N. S.
MCV (μm^3)	89.9±2.6	89.1±5.6	N. S.	88.9±5.2	N. S.	0.05
MCHC (%)	34.2±0.5	33.3±1.3	N. S.	33.3±1.3	N. S.	N. S.
Plt ($10^4/mm^3$)	22.6±4.2	21.1±3.1	N. S.	22.1±3.6	N. S.	N. S.
WBC ($10^3/mm^3$)	4.4±1.5	5.4±1.4	N. S.	5.0±1.0	N. S.	N. S.
RCF (%)	87.7±1.0	84.6±2.2	0.001	84.0±2.9	N. S.	N. S.

S2 = 23 female subjects before the menopause; Cont. = normal controls; P (Cont.-S2) =P value between Cont. and S2; S2+OPCs = S2 after the administration of OPCs; P (S2-S2+OPCs)= P value between S2 and S2+OPCs; P (S1-S2) = P value between S1 and S2. The data are expressed as means ± SD.
Ht, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cell; Plt., platelet; RCF, erythrocyte (red cell) filterability.

In our previous filtration study, we noted the impaired filterability of erythrocytes in smokers and subjects with metabolic syndrome such as mild obesity, hypertension, hyperlipidemia, or impaired glucose tolerance[4]. Metabolic syndrome is known to generate various kinds of reactive oxygen species and cause oxidative stress[20]. Our previous study further revealed a marked improvement in erythrocyte filterability in these subjects by oral OPCs supplementation, probably through its antioxidative action. Since OPCs have been attracting considerable public attention as an excellent supplement improving the quality of life, the study of the rheologic effects of OPCs is essential for basic, clinical, and nutritional science. The widespread effects of functional foods tend to be easily exaggerated without scientific investigations. Our successive studies clearly discriminated between the effectiveness and ineffectiveness of OPCs depending on the enrolled subjects with disturbed microcirculation based on the different rheologic mechanisms.

Since OPCs did not improve the impaired filterability in subjects with cold intolerance (Fig. 3, Tables 1 & 2), it is unlikely that the impaired filterability was due to oxidative injury to the erythrocyte membrane. Female subjects with cold intolerance tend to skip breakfast and dislike protein-rich foods[3]. This kind of malnutrition is suspected to reduce thermogenesis. In such a situation, peripheral vasoconstriction readily occurs in response to cold stress so as to prevent thermodiffusion[2]. Therefore, persistent vasoconstriction may easily cause mechanical stress in erythrocytes in subjects with cold intolerance. It is well known that several kinds of mechanical stress cause impairments in erythrocyte filterability through Ca^{2+}-dependent and cAMP-mediated signal transduction mechanisms[23)-25)]. It is therefore conceivable that in these subjects, there exists a vicious cycle of malnutrition causing impaired thermogenesis and persistent vasoconstriction leading to the mechanically induced impairment of erythrocyte fiterabiilty, which in turn induces the further disturbance of microcirculation and intolerance to cold stress. However, future studies are required to survey nutrition and investigate microcirculation and thermogenesis in these subjects with cold intolerance.

In conclusion, we observed an obvious impairment in the filterability of erythrocytes from subjects with cold intolerance, irrespective of menopause, and no rheologic effects of OPCs on the filterability, using the nickel mesh filtration technique. We speculate that some nutritional or metabolic problems may induce persistent peripheral vasoconstriction and mechanically impaired erythrocyte fiterabiilty in cold intolerance. Future rheologic studies will contribute to elucidate the pathophysiology of cold intolerance in more detail.

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