Original Article

The Decrease of Rouleaux Formation of Red Blood Cells in Healthy Human by Water-Soluble Chlorophyll as Revealed by Scanning Electron Microscopy

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ABSTRACT

Objective: In present study, we examined rouleaux formation of red blood cells before and after drinking water-soluble chlorophyll as an antioxidant. **Method:** In blood smear, red blood cells of 20 volunteers were examined before and after drinking water-soluble chlorophyll by using a light microscope, erythrocyte sedimentation rate (ESR) and scanning electron microscopy (SEM). **Results:** The rouleaux formations were modularly found in 30 and 60 min. In contrast, those formations rarely appeared in 45 min. ESR values of both sexes after drinking water-soluble chlorophyll for 45 min were significantly lower than those before drinking. In SEM, before drinking water-soluble chlorophyll, rouleaux formations were found. After drinking water-soluble chlorophyll for 45 min, however, those formations disappeared. **Conclusion:** The results suggest that water-soluble chlorophyll may be able to decrease rouleaux formations of red blood cells in healthy volunteers.

Keywords: water-soluble chlorophyll, rouleaux formation, red blood cell, scanning electron microscopy

Thai Pharm Health Sci J 2009;4(4):450-455[§]

Introduction

The rouleaux formation is stacks of red blood cells formed.[1] The flat surfaces of red blood cells give them a large area to make contact and stick to each other forming rouleux.[2] This formation is commonly found in multiple myeloma and macroglobulinemia disease, and is due to the presence of high concentration of abnormal globulins.[3,4] In healthy human, however, the rouleaux formation is also observed.[5] When red blood cells overlap, their surfaces for transportation of gases, hormone and nutrient are closed.[6] It is known that free radicals are the cause of the increase of rouleaux formation.[7] Free radicals that are originated from oxidation are atoms with unpaired electrons.[8] It is then necessary to neutralize themselves by taking electrons from cell membranes causing cell injury.[9] According to the free radical theory, radicals can damage cells in the organism, causing aging.[10] In human body, free radicals are controlled by enzymes such as glutathione peroxidase, superoxide dismutase and catalase.[11] In addition, antioxidants are substances that slow down or stop free radical oxidation in vivo.[12] The body can get antioxidants from food such as vitamin C, vitamin E, β-carotene, resveratrol, etc.[13,14] In the food from plant, human can get chlorophylls which are common material in plants. Chlorophylls are absorbed along human intestine.[15] Mario et al (2001) demonstrated the uptake of chlorophyll derivatives by human intestinal cells, and supported a potential role of chlorophylls as health-promoting phytochemicals.[16]

[§] 14th year of Srinakharinwirot Journal of Pharmaceutical Science

Chlorophyl derivatives are insoluble in water and difficult to be absorbed in human body.[17] Like vitamin C and vitamin E and other antioxidants, chlorophylls also have the antioxidant functions in the human body against free radicals.[16,18] Water-soluble chlorophyll is bland and non-irritating, and is used as antioxidant with a total absence of toxicity in clinical use.[19,20]

In this study, we examined the effect of antioxidant chlorophyll on the blood morphology of normal human specifically rouleaux formation before and after an intake of water-soluble chlorophyll.

Materials and Methods

Chemicals and reagents

Water-soluble chlorophyll was purchased from De Souza (California, USA). Wright stain and buffer pH 7.5 were purchased from CAMCO (Florida, USA). Potassium EDTA, glutaraldehyde, cacodylate buffer and osmium tetroxide were purchased from WAKO (Tokyo, Japan).

Volunteer enrollment

Healthy men and women with age of 18 to 21 years old were invited to be volunteers in this experiment. These volunteers were students in Srinakharinwirot University. All volunteers were required to provide written informed consent. This experiment design was approved by the ethics committee of Srinakharinwirot University Hospital, Thailand, in accordance with the recommendations of the Helsinki Declaration.

Preparation of Chlorophyll Solution and Blood Smear

Water-soluble chlorophyll was diluted with water (1:15) to a total volume of 150 mL to make solution. A 50 µL of blood was drawn from each volunteer. The volunteer was then given 10 mL of the chlorophyll solution orally. After the chlorophyll intake, blood sample was taken again at 30, 45, and 60 min. After chlorophyll intake, the volunteers were not allowed to drink any water or beverages.

Blood sample was dropped on slide, smeared and stained with Wright stain for 3 min. The smeared blood and Wright stain on slides were mixed with buffer pH 7.5 for 5 min and washed with distilled water. The slides of blood smear were observed for morphology under a light microscope (BX51 Olympus, USA).

Erythrocyte Sedimentation Method in Wintrob tube

Before and after drinking chlorophyll solution, 5 mL blood samples of 10 volunteers (5 men and women each) from venous puncture of the opposite arm were mixed with potassium EDTA for 10 min. Blood sample of each volunteer was injected to Wintrob tubes by needle syringe, and then kept at room temperature for 1 h. The ESR values were recorded at this time.

Scanning Electron Microscope

Before and 45 min after intake of chlorophyll solution, each blood sample (5 men or 5 women) was used for studying rouleaux formation by the scanning electron microscope. To prepare the blood sample slide, 50 mL of 2.5% v/v glutaraldehyde, as a fixative solution, was dropped on microscope slide. A blood sample of 20 µL of each volunteer was immediately dropped onto the fixative solution on the slide for 4 min. This blood sample was then soaked by cacodylate buffer for 2 hrs, and rinsed by 1% v/v osmium tetroxide for 5 min for 3 times, and finally washed by distilled water for 30 min. The blood sample was dehydrated through graded alcohol series, dried by critical point drying (K850 Emitech, England) for 10 min, and then coated with gold particles using sputter coater (K675X, England). These bloods samples before and after chlorophyll intake were examined morphologically under a scanning electron microscope (JSM-5400 JEOL, US).

Statistical analysis

For ESR data, the mean and standard deviations (SD) were calculated. In this study, the paired *t*-test was employed to examine difference of ESR between before and after chlorophyll intake (at 0 and 45 min). Analyses were performed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL).

Results

Before drinking water-soluble chlorophyll (0 min), the rouleaux formations were found to be moderate compared with those at 30, 45 and 60 min. At 30 min after chlorophyll intake, the rouleaux formation appeared to be low compared with those at 0, 45 and 60 min. Rouleaux formation was rarely found at 45 min after the intake; but after 60 min, however, the rouleaux could be observed again (Figure 1). Based on this finding, after drinking water-soluble chlorophyll for 45 min, this time-frame should be used in the following text of ESR and SEM analysis.

It was found that ESRs in men and women before drinking chlorophyll solution were 13.0 ± 0.4 and 17.2 ± 0.3 mm/hr, respectively. After chlorophyll intake for 45 min, ESRs in men and women decreased to 12.2 ± 0.5 and 15.9 ± 0.6 mm/hr, respectively (Table 2). This decrease was found statistically significant for both genders ($P < 0.05$).

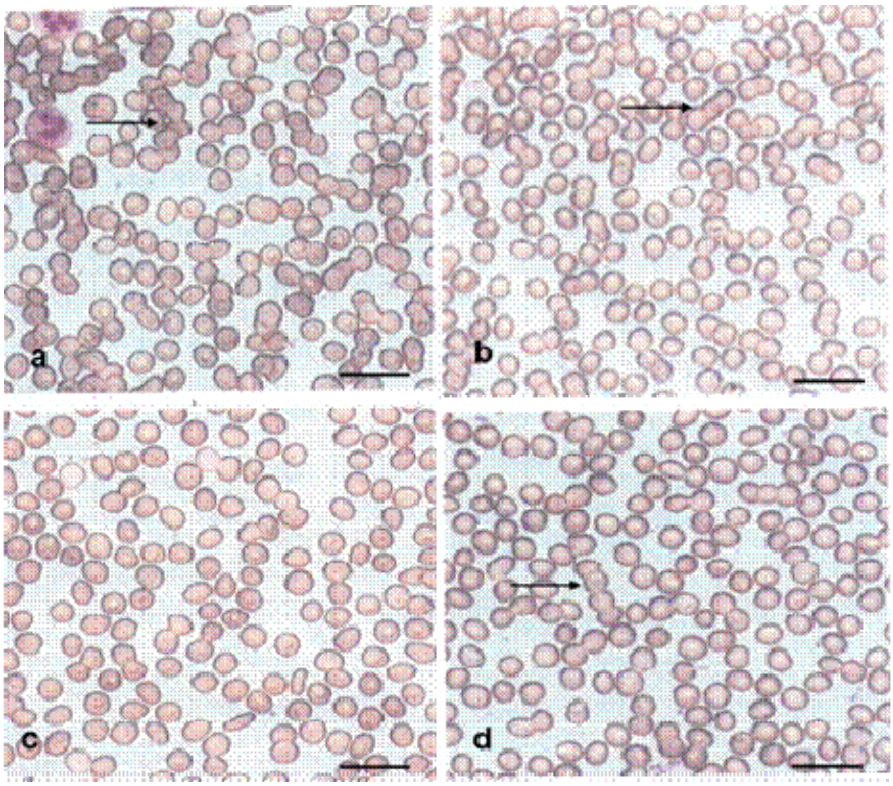

Figure 1 The photographs show the results of blood smear of healthy volunteers: (a) before drinking chlorophyll solution, and after drinking chlorophyll solution for (b) 30 min, (c) 45 min, and (d) 60 min. The arrows indicate rouleaux formation in blood samples. Bars indicate 25 μm.

Table 2 Erythrocyte sedimentation rates (ESRs) before and 45 min after intake of chlorophyll solution.

Gender	ESR (mm/hr) (mean ± SD)	
	Before intake	45 min after intake
Men (n = 5)	13.0 ± 0.4	12.2 ± 0.5*
Women (n = 5)	17.2 ± 0.3	15.9 ± 0.6*

* $P < 0.05$, between before and after chlorophyll intake (paired t-test)

Scanning electron microscope (SEM) results indicated that the rouleaux formation was evident before drinking chlorophyll solution (Figure 2A). After chlorophyll intake for 45 min, rouleaux formation disappeared and most red blood cells showed their surfaces (Figure 2B). In addition, morphology of red blood cells was normal.

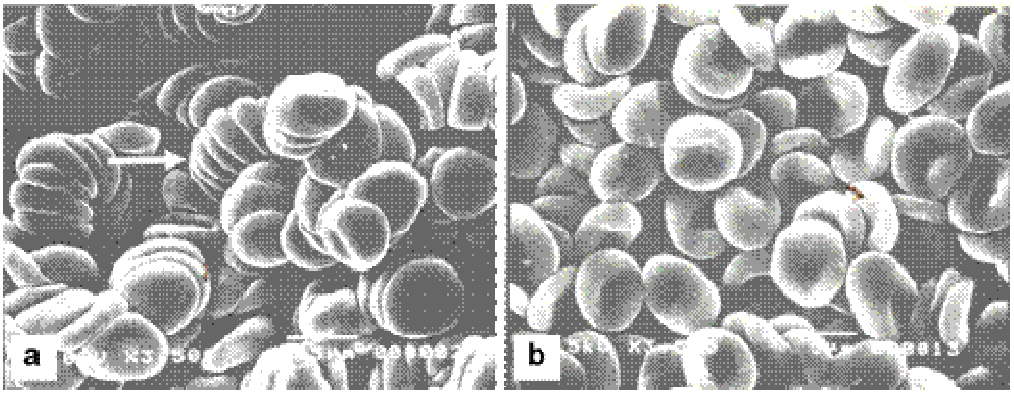

Figure 2 Photographs show the results of scanning electron microscope: (a) before drinking chlorophyll solution and (b) 45 min after chlorophyll intake. The arrow indicates rouleaux formation. Bars indicate 5 μm.

Discussion

Based on blood smear examination, the rouleaux formation in blood samples from 20 volunteers after drinking water-soluble chlorophyll for 45 min was found less and more singular red blood cells were observed. However, at 60 min after chlorophyll intake, rouleaux formations were again apparent. Therefore, the time that red blood cell was most affected by water-soluble chlorophyll intake is approximately 45 min.

Before drinking chlorophyll, the rouleaux formation was found moderate in the healthy volunteers. This finding was consistent with a report of Cerny et al (1988) that the rouleaux formations were moderately observed in healthy human.[21] It is known that, in blood smear observation, numerous rouleaux formations were presented in the patients with diseases caused by free radicals such as multiple myeloma.[21,22] Murina et al also showed that the rouleaux formations were increased after UV irradiation.[23] However, the rouleaux formation was decreased and prevented when treated with antioxidant phthalocyanines.[24] Furthermore, it is known that nitric oxide was antioxidant which reduced the development of rouleaux and the stacking of red blood cells in a large venule of mice.[25,26] Similar to our results, it was hard to find the rouleaux formation after intake of water-soluble chlorophyll. Therefore, results from ours and others support that water-soluble chlorophyll has potential as antioxidant to reduce rouleaux formation of red blood cells in human.

Marsh and Coombes reported that dietary supplementation of vitamin E and α-lipoic acid increased inhibition of the intrinsic coagulation pathway.[27] Negative charge such as free radicals affected the intrinsic coagulation pathway probably by activating this intrinsic pathway, which further caused the increase of ESR.[28] Moreover, the ESR was rapid in rouleaux network.[29] In this study, the values of ESR after drinking water-soluble chlorophyll were significantly lower than before the intake. Therefore, the low ESRs are evidence indicating that activity of antioxidant chlorophyll may reduce the rouleaux formation in healthy volunteers.

Formation results from both blood smear and Wintrob tubes were confirmed by scanning electron microscope images. Before chlorophyll intake, some rouleaux formations were observed. After the intake, however, such formation was resolved. There were more singular red blood cells of which surfaces were larger than those of found in the rouleaux formations. In general, singular red blood cells can carry the oxygen molecules for gas exchange.[30] In rouleax formation, however the clumping prevents oxygen molecules to be absorbed to the red blood cells, and transported, and thus tissue poor oxygenation. It thus suggests that water-

soluble chlorophyll may be an alternative antioxidant for promoting health and preventing the free radicals in human.

Conclusions

In summary, it can be suggested that drinking water-soluble chlorophyll can decrease the presence of rouleaux formations in healthy human. As far as our data show, however, the mechanism of inhibiting rouleaux formation by water-soluble chlorophyll seems to be not so simple but rather complicated. In the future, the mechanism of antioxidant-chlorophyll regulation on rouleaux formations mediated by free radicals should be evaluated.

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