Increased Band 3 Protein Aggregation and Anti-band 3 Binding of Erythrocyte Membranes on Treatment with Sesamol

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The influence of sesamol, an antioxidant in processed sesame oil, on oxidative modification of human erythrocyte membrane proteins was investigated. Human erythrocytes were incubated with sesamol at various concentrations up to 10 mM at 37 °C for 1 h. The amounts of hemoglobin bound to the membranes and detergent C12E8-insoluble membrane protein aggregates were increased as the concentration of sesamol increased. Western blot analysis indicated that aggregates of band 3 protein were increased by the treatment. Binding of anti-band 3 antibody to the erythrocytes was increased by the treatment. Isolated cell membranes were incubated with sesamol similarly. Aggregates of band 3 protein were also increased, indicating that the band 3 protein aggregation was little affected by hemoglobin bound to the membranes. Aggregation of band 3 protein in the treatment of isolated cell membranes was partially prevented when the treatment was conducted under anaerobic conditions, suggesting that augmentation of the protein aggregation by sesamol involved both oxygen-dependent and oxygen-independent pathways. Among phenolics, sesamol showed a distinctive feature to increase band 3 protein aggregation in erythrocyte membranes and to enhance anti-band 3 binding to erythrocytes.

Key words sesamol; erythrocyte; protein aggregation; band 3; anti-band 3 binding

Processed sesame oil, widely used as an edible oil and as a solvent for injections, contains several constituents including sesamin, sesamolin, sesamol and sesaminol.1–3) Certain processing of sesame oil results in the formation of sesamol from its bound form sesaminol.4,5) While sesamol is a potent phenolic antioxidant contained only in processed sesame oil,1,2) very little is known about its biological effects. In the course of our investigation on sesamol, it has been found that sesamol converts hemoglobin into methemoglobin (MetHb) in human erythrocytes, and its activity is more extensive than those of other phenolics.6,7)

During circulation in human body, erythrocytes undergo oxidative aging. Senescent erythrocytes undergo various oxidative modifications of cellular components, formation of oxidatively denatured hemoglobin,8,9) peroxidized lipids,10) fluorescent chromolipids,11,12) high-molecular weight cross-linked membrane proteins,13,14) cross-linked complex of spectrin and hemoglobin15) and aggregates of band 3 protein.8,16) Our previous study showed that anti-band 3 autoantibody binds to in vitro oxidized erythrocytes17) and also to in vivo senescent erythrocytes,18) and this event is caused by aggregation of band 3 protein.16)

The aim of the present study was to clarify whether sesamol affected the oxidative or aging modifications of erythrocyte membranes, band 3 protein aggregation and anti-band 3 binding. It was found that sesamol showed a distinctive feature to increase band 3 protein aggregation in erythrocyte membranes and to enhance anti-band 3 binding to erythrocytes.

MATERIALS AND METHODS

Materials  Sesamol was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI) and was recrystallized from chloroform–petroleum ether.19) (+)-Catechin, (+)-epicatechin, globulin-free bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Protein A-horseradish peroxidase conjugate was obtained from Bio-Rad Laboratories (Richmond, CA). NaCl was obtained from ICN Pharmaceutical (Irvine, CA). Clear Blot Membrane-P, a polyvinylidene difluoride (PVDF) microporous membrane for protein blotting, was obtained from Atto Co. (Tokyo). Octaethylene glycol n-dodecyl monoether (C12E8) was obtained from Nippon Chemical Co. (Tokyo). Gallic acid p-propyl ester was purchased from Tokyo Chemical Industry Co. (Tokyo). Quercetin dihydrate, (−)-epigallocatechin gallate, and other reagents were purchased from Wako Pure Chemical Industries (Osaka).

Preparation of Erythrocytes and Ghosts  Human blood withdrawn from a healthy donor using citrate-phosphate-dextrose as an anticoagulant was stored at 4 °C for use within 4 d. Erythrocytes were collected from blood by centrifugation and washed using standard procedures as described previously.16) Erythrocyte ghosts were prepared by the method of Dodge et al.20) and protein concentration of ghosts were determined by the Lowry method.21) For preparation of hemoglobin-free ghosts, cells were lysed and ghosts were centrifuged down and washed several times until the color of hemoglobin in the supernatant was diminished. Hemoglobin content was determined by absorbance at 523 nm and its molecular extinction coefficient of 7880.17) Erythrocyte volume was calculated using mean hemoglobin concentration of 339 g/l erythrocyte.22)

Treatment of Erythrocytes with Sesamol and Other Phenolics  A 40% erythrocyte suspension (5 ml) in Dulbecco's phosphate-buffered saline (DPBS) was mixed with an equal volume of various concentrations of sesamol or a phenolic in DPBS and the mixture was incubated under aerobic conditions at 37 °C for 1 h. The reaction mixture was then centrifuged at 650×g for 5 min to remove the supernatant, and the cell pellet was washed three times with Ca2+, Mg2+-free DPBS (DPBS(−)) and resuspended in an appropriate buffer for use.

Treatment of Erythrocyte Ghosts with Sesamol  Erythrocyte ghosts at 3 mg protein/ml in 3 ml DPBS were mixed with an equal volume of various concentrations of sesamol in...
DPBS and the mixture was incubated under aerobic or anaerobic conditions at 37 °C for 1 h. The reaction mixture was centrifuged to remove the supernatant and the pellet was washed three times with DPBS and two times with isotonic saline. In the reaction under anaerobic conditions, both the ghost suspension and the sesamol solution were separately deoxygenated by purging nitrogen gas for 1 h in airtight vessels. The suspension and the sesamol solution were mixed and the mixture was incubated in the airtight vessel. The reaction mixture was immediately transferred to a centrifuge tube and centrifuged to remove the supernatant. The pellet was washed three times with deoxygenated DPBS and twice with isotonic saline.

Cytoplasmic MetHb and Membrane-Bound Hemoglobin Cytoplasmic MetHb was measured according to the method previously described. The amount of membrane-bound hemoglobin including oxyhemoglobin (OxyHb), MetHb and denatured hemoglobin was determined by the pyridine hemochromogen method as described previously.

Isolation of Protein Aggregates in Erythrocyte Membranes Nonionic detergent C12E8-insoluble protein aggregates in erythrocyte membranes were isolated according to the method previously described. After washing, the cells were lysed, and the ghosts were collected. Ghosts were successively treated with EDTA to prepare inside-out vesicles. C12E8 was added to the vesicles, and the mixture was centrifuged at 70000×g for 1 h. C12E8-insoluble membrane proteins were obtained as aggregates in the pellet. The aggregates were dissolved into 0.5 ml of a solution of 2% sodium dodecyl sulfate (SDS), and protein content was determined by the method of Lowry et al.

Detection of Band 3 Protein in Protein Aggregates by Western Blotting The whole C12E8-insoluble aggregates isolated from the cell membranes were solubilized in 0.5 ml of 2% SDS, and mixed with an equal volume of electrophoresis buffer. A 10-μl aliquot of the solution was subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reduced or unreduced conditions. SDS-PAGE was performed according to the methods of Laemmli using 10% polyacrylamide gel. The gel was stained by Coomassie brilliant blue R-250 (CBB) or subjected to Western blotting according to the method of Towbin et al.

Band 3 protein in the aggregates transferred on a PVDF membrane was detected according to the method described previously. Briefly, after blocking the PVDF membrane with 1% BSA, the membrane was incubated with affinity-purified rabbit anti-human band 3 IgG at room temperature for 2 h, followed by incubation with protein A-horseradish-peroxidase conjugate. The peroxidase activity was detected with hydrogen peroxide and 4-chloro-1-naphthol.

Binding of Autologous IgG to Sesamol-Treated Erythrocytes Autologous IgG was purified from plasma of the same donor as described. Autologous IgG binding assay was performed according to the method described. Sesamol-treated and untreated erythrocytes were resuspended in 1% BSA in DPBS(−) at a concentration of 40%. An erythrocyte suspension (10 μl) was incubated with a 100-μl solution of autologous IgG (5 mg/ml) in DPBS(−) at 4 °C overnight. After washing the cells four times with DPBS(−), they were resuspended in 100 μl of the same buffer. The amount of cell-bound IgG was measured by enzyme immunoassay using a protein A-β-galactosidase conjugate. The data are expressed as the cell-bound β-galactosidase activity, where 1 unit is defined as the activity that hydrolyzes 1 μmol of the substrate per 1 min under the conditions employed.

Binding of 125I-Labeled Anti-band 3 IgG to Sesamol-Treated Erythrocytes Human anti-band 3 IgG was isolated from normal adult plasma by anion exchange chromatography, and anti-band 3 IgG was purified from IgG fraction by affinity chromatography using a band 3-coupled Sepharose gel column by the method of Lutz et al. as described previously. 125I-Labeling of anti-band 3 IgG was carried out by the chloramine T method.

RESULTS

Increased Binding of Hemoglobin to Erythrocyte Membranes on Sesamol Treatment Effect of sesamol treatment of erythrocytes on the cytoplasmic OxyHb transformation into MetHb and binding of hemoglobins including OxyHb, MetHb and other denatured hemoglobin to the cell membranes was examined. Erythrocytes were treated with 0.5—10 mM sesamol at 37 °C for 1 h (Table 1). The amount of cytoplasmic MetHb was increased as the concentration of sesamol increased, and almost 100% OxyHb was converted into MetHb on 5 mM sesamol treatment. The amount of membrane-bound hemoglobins as assessed by the pyridine hemochromogen methods was found to be increased as the concentration of sesamol increased, and the amount of hemoglobins bound to the membranes of the cells treated with 10 mM sesamol was 20-fold that of untreated cells. It is likely that binding of hemoglobins to the cell membranes is caused by the formation of MetHb or other denatured hemoglobins.

<table>
<thead>
<tr>
<th>Sesamol (mM)</th>
<th>MetHb* in cytosol (% of total hemoglobins)</th>
<th>Membrane-bound hemoglobin* (mg/mg of ghost protein)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>7.1</td>
<td>0.036</td>
</tr>
<tr>
<td>0.5</td>
<td>59.7</td>
<td>0.034</td>
</tr>
<tr>
<td>1</td>
<td>79.8</td>
<td>0.033</td>
</tr>
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<tr>
<td>5</td>
<td>100</td>
<td>0.565</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.765</td>
</tr>
</tbody>
</table>

*a* Incubated at 37 °C for 60 min. *b* After hypotonic hemolysis of erythrocytes, cytoplasmic MetHb was measured spectrophotometrically using a molar extinction coefficient of 40100 (on a hemoglobin basis) at 630 nm. MetHb content was expressed as the percent of total hemoglobins which was determined by absorbance of the hemolysate at 523 nm, an isobestic point of OxyHb and MetHb (a molar extinction coefficient of 7880 on a hemoglobin basis). c Erythrocytes were lysed and ghosts were collected. Protein content in the ghosts was determined by the Lowry method. Membrane-bound hemoglobin content in the ghosts was measured by the pyridine hemochromogen method.
Several attempts were made in order to find evidence for generation of any oxidative species in the interaction of hemoglobin with sesamol under aerobic conditions. A mixture of 0.1% hemolysate or 1 mM FeSO₄, and 10 mM sesamol was incubated in the presence of the spin trapping agent 5,5-dimethyl-1-pyrroline N-oxide at room temperature for 1 h. No electron spin resonance (ESR) signals were observable (data not shown). A mixture of 2% hemolysate and 1 mM sesamol was incubated at room temperature for 30 min to convert 92% Hb into MetHb. When this incubation was conducted in the presence of superoxide dismutase (0.1 mg/ml) + catalase (0.1 mg/ml), 1 mM Trolox, 0.1 mM nordihydroguaiaretic acid, and 0.1 mM propyl gallate, 93, 75, 75 and 62% OxyHb was converted into MetHb. Superoxide and hydrogen peroxide are not likely to be involved, and other oxidative species may participate little in the MetHb formation.

**Band 3 Protein Aggregation in the Cell Membranes of Erythrocytes Treated with Sesamol** We investigated whether band 3 aggregation was increased by the sesamol treatment. Erythrocytes were treated with 0.5—5 mM sesamol at 37°C for 1 h, and ghosts were prepared from the treated cells. Nonionic detergent C₁₅E₅-insoluble membrane proteins were obtained as aggregates. Protein content in the aggregates was increased as the concentration of sesamol increased, and the content obtained on 5 mM sesamol treatment was approximately 6-fold that of untreated cells (Fig. 1). The whole aggregates were subjected to SDS-PAGE and stained by CBB. The protein aggregates obtained from the membranes treated with 5 mM sesamol showed several protein bands on the gel (Fig. 2A, lanes f and k), indicating that the aggregates were constituted of noncovalently associated several membrane proteins. The membrane protein aggregates of the cells treated with 5 mM sesamol showed the band of hemoglobin at the bottom of the electrophoresis, hemoglobins were included in the aggregates. The amount of band 3 protein in the aggregates was increased as the concentration of sesamol increased, as analyzed by Western blotting (Fig. 2B). Most of band 3 protein in the aggregates was in a monomer form, but high-molecular-weight band 3 protein was also present indicating that band 3 protein associated tightly itself or formed complex with other membrane proteins. Because the electrophoretic profiles under reduced and unreduced conditions were similar, the membrane protein aggregation may be due to disulfide-independent crosslinking.

**Involvement of Hemoglobin Binding to Erythrocyte Membranes in the Band 3 Protein Aggregation Increased by Sesamol Treatment** Whether or not binding of hemoglobins to the inner surface of erythrocyte membranes was required to form aggregates of membrane proteins was investigated. Hemoglobin-free ghosts were incubated with sesamol at 37°C for 1 h under aerobic conditions. Protein content in C₁₅E₅-insoluble protein aggregates increased as the concentration of sesamol increased (Fig. 3). SDS-PAGE analysis of the protein aggregates (Fig. 4A) showed a profile similar to that obtained from the sesamol-treated erythrocytes. In this case, the band for hemoglobin was not observable. Because the electrophoretic patterns obtained under reducing and unreducing conditions were slightly different, disulfide bond formation may be included. The amount of band 3 protein in the aggregates was increased as the concentration of sesamol increased (Fig. 4B). This result indicates that aggregation of the membrane proteins containing band 3 protein can be increased without MetHb or denatured hemoglobins.

**Involvement of Oxygen in the Band 3 Protein Aggregation by Sesamol Treatment** Involvement of oxygen in the
band 3 protein aggregation increased by sesamol was investigated. Hemoglobin-free ghosts were treated with 10 mM sesamol under anaerobic conditions. Protein content in the C_{12}E_4-insoluble aggregates was slightly lower than that obtained under aerobic conditions (Fig. 5). Analysis of the aggregates by SDS-PAGE (Fig. 6A) and by subsequent Western blotting (Fig. 6B) showed that band 3 protein content in the aggregates under anaerobic conditions was slightly less than that obtained under aerobic conditions. This result indicated that the band 3 protein aggregation increased by sesamol partly depends on molecular oxygen. An extensive amount of

Fig. 3. C_{12}E_4-Insoluble Protein Aggregates in Sesamol-Treated Ghosts

Ghosts (1.5 mg/ml, 6 ml) were incubated with the indicated concentrations of sesamol at 37°C for 1 h. C_{12}E_4-insoluble membrane proteins were determined. The data are expressed as the mean±S.D. of triplicate experiments.

Fig. 4. SDS-PAGE Analysis of C_{12}E_4-Insoluble Aggregates Isolated from Sesamol-Treated Ghosts

The whole C_{12}E_4-insoluble aggregates isolated from the cell membranes (Fig. 3) were subjected to SDS-PAGE under reduced or unreduced conditions. The gels were stained with CBB (A), or subjected to Western blotting using rabbit anti-band 3 IgG (B). Lane: a, cell membrane proteins (0.5 mg/ml, 10 μl); b and g, control ghost aggregates; c and h, sesamol-treated (1 mM) ghost aggregates; d and i, sesamol-treated (2 mM) ghost aggregates; e and j, sesamol-treated (5 mM) ghost aggregates; f and k, sesamol-treated (10 mM) ghost aggregates. Arrows indicate the position of band 3 (monomer).

Fig. 5. C_{12}E_4-Insoluble Protein Aggregates Isolated from Ghosts Incubated with Sesamol under Aerobic (□) or Anaerobic (■) Conditions

Ghost (3 mg/ml, 3 ml) suspension and an equal volume of 20 mM sesamol solution were mixed and incubated under aerobic or anaerobic conditions at 37°C for 1 h. C_{12}E_4-insoluble membrane proteins were determined. The data are expressed as the mean±S.D. of triplicate experiments.

Fig. 6. SDS-PAGE Analysis of C_{12}E_4-Insoluble Aggregates Isolated from Ghosts Incubated with Sesamol under Aerobic or Anaerobic Conditions

The whole C_{12}E_4 insoluble aggregates isolated from the cell membranes (Fig. 5) were subjected to SDS-PAGE under reduced or unreduced conditions. The gels were stained with CBB (A), or subjected to Western blotting using rabbit anti-band 3 IgG (B). Arrows indicate the position of band 3 (monomer).
band 3 protein aggregates was produced even in the absence of molecular oxygen. The band 3 protein aggregation by sesamol may be caused by both oxygen-dependent and oxygen-independent pathways.

**Autologous IgG and Anti-band 3 IgG Binding to Sesamol-Treated Erythrocytes** Binding of autologous IgG to erythrocytes treated with various concentrations of sesamol was investigated by enzyme immunoassay. IgG binding was increased as the concentration of sesamol increased (Fig. 7A), and the amount of IgG bound to the cells treated with 5 mM sesamol was approximately 3-fold that to untreated cells. Human anti-band 3 IgG labeled with \(^{125}\)I binding was increased as the concentration of sesamol increased (Fig. 7B), and the amount of the bound anti-band 3 IgG to the cells treated with 1 mM sesamol was approximately 7-fold that to the untreated cells. The increase in the binding of autologous IgG and anti-band 3 IgG to the cells was correlated with the increase in the amount of band 3 protein aggregates in the cell membranes.

**Aggregation of the Membrane Proteins in Erythrocytes by Phenolics Other than Sesamol** Whether phenolics other than sesamol can increase protein aggregation in erythrocyte membranes was examined. Erythrocytes were treated with catechin, epicatechin, epigallocatechin gallate, propyl gallate and quercetin at 1–5 mM at 37°C for 1 h. C\(_{12}\)E\(_{4}\)-insoluble protein aggregates were determined (Table 2). The amount of aggregates obtained from 5 mM quercetin-treated cells was slightly increased, but other phenolics did not increase the aggregates in the cell membrane proteins. This result indicates that sesamol has a distinctive feature to increase protein aggregation in erythrocyte membranes and this activity is not commonly observed in other phenolic compounds.

**DISCUSSION**

Human erythrocytes undergo various oxidative damage in the circulation.\(^8\)–\(^{15}\) By oxidative stress, band 3 protein is aggregated in the membranes during the aging of erythrocytes in the circulation.\(^8\)\(^,\)^\(^{16}\) In turn, anti-band 3 antibody binds to the cells\(^7\)\(^,\)^\(^{18}\) through the aggregated poly-lactosaminyl sugar chains of band 3 protein on the cell surface.\(^16\)\(^,\)^\(^{27}\)\(^,\)^\(^{29}\) In the present in vitro study, it was found that sesamol among phenolics showed the distinctive feature to increase band 3 protein aggregation in erythrocyte membranes and to enhance anti-band 3 binding to erythrocytes. The present result may give another line of evidence showing the binding of the anti-band 3 antibody to the clustered cell surface band 3 protein.

Membrane proteins containing band 3 protein have been suggested to be clustered locally on erythrocyte surface by attachment of oxidized and denatured hemoglobin to the inner surface of the cell membranes.\(^{59}\) Low and his colleagues\(^\text{\textsuperscript{51}}\) have isolated co-clustered protein complexes containing denatured hemoglobins, band 3 and other membrane proteins from senescent erythrocytes and erythrocytes of abnormal hemoglobin disease. It has been shown that treatment of erythrocytes with sesamol in vitro results in the formation of MetHb.\(^6\)\(^,\)^\(^{52}\) In the present study it was found that binding of hemoglobin to the membranes was induced by sesamol treatment of the cells. It is likely that the binding was caused by MetHb and other denatured hemoglobins. However, binding of hemoglobins to the cell membranes in the sesamol treatment was not requisite for membrane protein and band 3 aggregation, because the membrane protein aggregation was induced by sesamol treatment of hemoglobin-free ghosts. Sesamol-induced protein and band 3 aggregation was found to be an independent-event from MetHb formation.

It has been shown that sesamol produces MetHb from OxyHb and even from deoxyhemoglobin (DeoxyHb), but does not reduce MetHb.\(^6\)\(^,\)^\(^{52}\) Participation of oxidation process in the MetHb formation was not shown in the present study. Stronger reductant polyphenolics such as pyrocatechol, p-hydroquinone and pyrogallol moderately produce MetHb from.

### Table 2. Aggregation of Membrane Proteins in Erythrocytes by the Treatment of Phenolics

<table>
<thead>
<tr>
<th>Erythrocytes treated with[(\text{a)}]</th>
<th>Protein aggregates (ratio to control[(\text{b)}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.00</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.78±0.03</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>1.02±0.05</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>0.95±0.07</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>1.26±0.06</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.53±0.08</td>
</tr>
</tbody>
</table>

[\(\text{a)}\) Erythrocytes were incubated with the indicated concentrations of each phenolic at 37°C for 60 min. C\(_{12}\)E\(_{4}\)-insoluble membrane proteins were determined. [\(\text{b)}\) Results are expressed as the mean±S.D. of triplicate determinations.

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**Fig. 7.** Binding of Autologous IgG (A) and Anti-band 3 IgG (B) to Erythrocytes Treated with Sesamol.

Erythrocytes were incubated with the indicated concentrations of sesamol at 37°C for 1 h. A: Cells were incubated with autologous IgG at 4°C overnight. The data are expressed as the mean±S.D. of the cell-bound β-galactosidase activity in triplicate determinations. B: Cells were incubated with \(^{125}\)I-labeled anti-band 3 IgG at 4°C overnight. The cell-bound radioactivity was counted. The data are expressed as the mean±S.D. of ng of \(^{125}\)I-anti-band 3 IgG bound per million of the cells in triplicate determinations. The amount of the cells was corrected by calculation of hemoglobin content of the hemolysate of the sample cells.
OxyHb but not from DeoxyHb, and effectively reduce MetHb. The action mechanisms of the di and triphenolics have been explained as follows. OxyHb might exist in a transition state as a superoxoferrihemoglobin complex, and donation of one electron from the di- and triphenolics to the bound superoxide would result in the production of MetHb, hydrogen peroxide and phenoxy radical. The action mechanisms of sesamol could not be explained by the reaction sequences illustrated for the stronger reductants. The action of sesamol may be explained by considering its interaction with the globin moieties of hemoglobin. Interaction of sesamol with the globin moieties might induce intramolecular electron transfer in DeoxyHb molecule to form MetHb.

It is interesting to note that antioxidant sesamol did not prevent oxidative modifications of membrane proteins of erythrocytes, but caused membrane protein aggregation. Band 3 aggregation in the erythrocyte membranes induced by sesamol was only slightly decreased in the absence of molecular oxygen. Most part of the aggregation may be induced without the aid of molecular oxygen. Action mechanisms of sesamol for the protein and band 3 aggregation may be due to the direct denaturation of the membrane proteins and the cytoskeleton proteins to cause denaturation and aggregation of the whole membrane proteins. The whole membrane proteins may be co-clustered in the membranes and as a consequence band 3 protein may be clustered in the membranes.

Sesamol, an antioxidant of sesame oil, was found to induce MetHb formation and membrane protein aggregation in erythrocytes even under anaerobic conditions in the present in vitro study. Sesamol has the distinctive feature for MetHb formation and membrane protein aggregation among the phenolic antioxidants. Although sesamol is an effective antioxidant in sesame oil, it may give unbenefficial effects of direct denaturation of hemoglobin and membrane proteins on erythrocytes. In vivo studies are necessary to clarify the effects of sesamol on erythrocytes.

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REFERENCES