Clinical Evaluation of Alpha-Tocopherol in Buccal Mucosal Cells of Children

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Summary 1. Tocopherol concentrations in plasma, red blood cells (RBCs), and buccal mucosal cells were examined in newborn infants (before feeding), children (2–15 years old), and adults. Tocopherol concentrations in adults and newborn infants showed the greatest difference in plasma and the smallest difference in RBC. Buccal cell tocopherol concentrations in adults were 2.3 times higher than those in infants. The majority of newborn infants had RBC tocopherol levels below the normal limit (115 μg/100 ml of packed cells). Also, more than one third of buccal cell tocopherol levels determined in newborn infants were below a level of 15 ng/mg protein, which was determined as the lower limit of normal in healthy children on the basis of the testing of 97 samples. 2. After administration of a daily dose of 600 mg of RRR-alpha-tocopherol for three months to young adults, tocopherol levels in buccal mucosal cells reached a level of more than 4 times the basal level after rising throughout the 3-month period, while levels in RBC and plasma showed less than a 3-fold increase and reached a maximum within one month. Buccal cell tocopherol levels showed a poor correlation to the RBC and plasma tocopherol levels, while the tocopherol/lipid ratio was closely correlated with RBC and plasma tocopherol levels. After a single dose of 600 mg of RRR-alpha-tocopherol, RBC and plasma tocopherol concentrations reached a maximum within 24 hours, while buccal cell tocopherol did so 4 to 6 days later. 3. Very obese children and hyperlipemic obese children showed lower buccal cell tocopherol levels, accompanied by lower RBC tocopherol levels and higher plasma tocopherol levels, when compared with non-obese children and normolipemic obese children.

Key Words vitamin E, buccal mucosal cell, erythrocyte, plasma, platelets infant, obesity, hyperlipemia
For the assessment of human vitamin E status, several newly proposed indices have been reported including the plasma tocopherol/lipid ratio (1-3), the platelet tocopherol (4-7) and the red blood cell (RBC) tocopherol level (8-10), because the commonly used plasma tocopherol level has been shown to be easily affected by variations in plasma lipids (11-13). However, these indices do not give consistent results regarding the nutritional status of vitamin E in children, especially in neonates (14). The recent progress in HPLC has facilitated the assay of very small amounts of tocopherol, allowing tocopherol levels in swabbed mucosal cells to be determined, and the suitability of buccal cells for assessing tocopherol levels has been investigated (15). In this study, we investigated buccal cell tocopherol concentrations in neonates, children, and adults, as well as the changes in buccal cell tocopherol concentrations after the oral administration of a vitamin E preparation in relation to the changes in RBC and plasma tocopherol levels. In addition, the effects of body lipid pools on the biological availability of tocopherol in vivo were investigated on the basis of changes in the buccal cell tocopherol concentration.

MATERIALS AND METHODS

Subjects. 1) Newborn infants: Nineteen healthy mature newborn infants (weighing 2,750 to 3,680 g) from our newborn nursery were examined before their first feed after birth. None of the infants had delivery complications (Apgar score was more than 8) or any evidence of intrauterine disturbances.

2) Children: Ninety-seven apparently healthy children (2 to 15 years old) (54 boys and 43 girls) who were on an adequate diet but no vitamin E supplements, and who visited our outpatient clinic for health checks, were enrolled.

3) Adults: Healthy male volunteers, aged from 23 to 27 years old, were enrolled in this study. The adult volunteers were administered a daily dose of 600 mg of RRR-alpha-tocopherol for 3 months.

4) Obese children: Fifty-five obese children (32 males and 23 females, with various degrees of obesity) who were attending our outpatient clinic for obesity were also enrolled in this study. The tocopherol concentration was compared between children with different degrees of obesity. Furthermore, 21 of 74 male obese children with plasma lipid levels of 500 mg or more/100 ml were compared with 38 age-matched obese children with plasma lipid levels under 500 mg/100 ml. The grade of obesity was calculated as the percentage obesity for height.

The study protocol was approved by the ethics committee at the college hospital, and the study was performed after informed consent was obtained from the parents and subjects.

Sample preparation. 1) Buccal mucosal cells: The subjects first rinsed their mouths with distilled water, and the buccal mucosal cells were then collected by gentle scraping with a spatula. Cells on the spatula were suspended in isotonic saline and washed three times with saline. After the last washing, the pellet was resuspended in 2 ml of distilled water. Then, the suspension was vortexed and
sonicated at 20 Kc for 30 sec. Aliquots of 1 ml and 0.4 ml were separately taken up for tocopherol and protein assays, respectively. In the newborn infants, buccal cells were directly collected without rinsing with distilled water.

2) RBCs, and plasma: Heparinized blood was collected by venipuncture at the same time as the sampling of the buccal cells. Preparation of plasma, RBCs, and platelets was carried out as described in previous reports (8-10).

Analysis. 1) Buccal cells: The method for tocopherol assay in buccal mucosal cells has been described in our previous report (15). In brief, 1 ml of the buccal cell suspension was placed into a centrifuge tube together with 1 ml of 6% pyrogallol solution in ethanol, and 1 ml of all-rac-tocol solution in ethanol (2 µg/ml) as an internal standard. Cells were then preincubated for 2 min at 70°C. After preincubation, the solution mixture was added to 0.2 ml of 60% KOH and saponified at 70°C for 30 min. Following the saponification, the sample solution was immediately cooled with water, mixed with 2.5 ml of distilled water and 5 ml of n-hexane, and then vigorously shaken for 5 min. The mixture was centrifuged at 3,000 rpm for 5 min to obtain a hexane layer, and 4 ml of this hexane layer was evaporated under nitrogen at 40°C. The residue was dissolved in 50 µl of ethanol, and a 15-µl sample was injected into the HPLC column. The instruments used in this assay were as follows: the HPLC equipment was an IRIKA P-530 (IRIKA Co. Ltd., Kyoto) with an IRIKA RP-18 4 × 250 mm column. Eluents were methanol/water/NaClO₄ at a ratio of 1000/2/7 (v/v/w). Flow rate was 10 ml/min. The detector was an IRIKA Anpreometric E-520 detector. Retention time was 6.88 min for tocol, and 7.78 min for delta-, 9.36 min for gamma- and beta-, and 10.88 min for alpha-tocopherol. In this paper, only the results for alpha-tocopherol are given. Protein content was determined using a 0.4 ml aliquot of the cell suspension by the method of Lowry et al. (16). The buccal cell tocopherol levels were expressed as ng/mg protein.

2) RBCs, and plasma: Tocopherols were extracted from the RBCs and plasma, which were prepared as described in previous reports (8-10). For the assay, another HPLC system was used; Shimadzu LC-5A (Shimadzu Co. Ltd., Kyoto) HPLC apparatus, with a NUCLEOSIL-5NH₂ 4 × 5 mm precolumn and a 4 × 150 mm main column. The eluent was n-hexane/isopropyl alcohol at a ratio of 97/3, the flow rate was 1.5 ml/min, and the detector was a Shimadzu RF-530 fluorospectrophotometer (Ex 298 nm, Em 325 nm). Retention time was 2.39 min for alpha-, 3.44 min for beta-, 3.68 min for gamma-, and 5.20 min for delta-tocopherol, and 5.96 min for tocol. The RBC tocopherol values obtained from HPLC analysis were corrected by the hematocrit values for each sample. In this report, only values for alpha-tocopherol are given.

Total cholesterol, phospholipids, and triglycerides were measured by the methods of Allein et al. (17), Takayama et al. (18), and Bucolo and David (19), respectively. Total lipids were estimated as the sum of these three major lipids.
RESULTS AND DISCUSSION

1. Tocopherol concentrations in the buccal mucosal cells of newborn infants, children, and adults, in comparison with plasma and RBC concentrations

Table 1 shows the tocopherol concentrations in buccal mucosal cells, RBC, and plasma as well as the tocopherol/lipid ratio for newborn infants, children, and adults. There were only small but significant differences in the tocopherol concentrations in RBC and plasma and the tocopherol/lipid ratios between children and adults. In contrast, tocopherol concentrations in all the samples from the newborn infants were generally much lower than in those from the adults. The concentrations in adult buccal cells, RBC, and plasma were, respectively, 2.3 times, 1.3 times, and 2.9 times higher than in newborn infants. The tocopherol/lipid ratio was 1.7 times higher in adults than in newborn infants. The difference was most marked in the plasma and was smallest in RBC. Plasma tocopherol levels in all of the newborn infants were lower than 450 µg/100 ml, while only 1 neonate had an RBC tocopherol level of less than 115 µg/100 ml packed cells. Only 2 of the 19 neonates showed values of less than 0.8 for the tocopherol/lipid ratio. The tocopherol levels of 450 µg/100 ml for plasma and 115 µg/100 ml packed cells for RBCs (9), and a tocopherol/lipid ratio of 0.8 (mg/g) (1) were taken as the respective lower limits of normal. The finding that plasma tocopherol levels were very much lower in newborn infants, while most of the RBC tocopherol levels and tocopherol/lipid ratios were within the normal adult range, was consistent with our previous reports which have suggested that no vitamin E deficiency exists in most premature infants (9,10). However, tocopherol concentrations in the buccal mucosal cells of newborn infants were less than half of the adult levels. The distribution of buccal cell tocopherol concentrations in neonates is shown in Fig. 1 in comparison with that in children. As shown in this figure, the distribution in neonates was generally shifted to lower levels than that in children. When the influence of sex was examined in the children, no differences were found in tocopherol levels between boys and girls (data not shown). The mean adult level in the buccal mucosal cells was 43.6 ng/mg protein, which agreed with the value of 46.4 ng/mg protein obtained in our previous preliminary study (15). On the basis of

Table 1. Tocopherol concentrations in buccal mucosal cells, RBCs, and plasma in newborn infants, children, and adults.

<table>
<thead>
<tr>
<th></th>
<th>Buccal mucosal cells (ng/mg protein)</th>
<th>RBCs (µg/100 ml packed cells)</th>
<th>Plasma (µg/100 ml)</th>
<th>Tocopherol/lipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn infants</td>
<td>19 19.3±10.1**</td>
<td>176.3±41.8**</td>
<td>264.2±86.7**</td>
<td>1.05±0.21**</td>
</tr>
<tr>
<td>Children</td>
<td>97 42.6±17.3</td>
<td>201.0±33.5**</td>
<td>686.7±164.3*</td>
<td>1.54±0.27**</td>
</tr>
<tr>
<td>Adults</td>
<td>19 43.6±9.6</td>
<td>226.3±19.2</td>
<td>772.5±165.1</td>
<td>1.76±0.14</td>
</tr>
</tbody>
</table>

Values are the M±SD, **p<0.01, *p<0.05 (vs the adult group).

Fig. 1. Distribution of tocopherol concentrations in buccal mucosal cells, plasma, and RBCs in newborn infants (lower panels), as compared with that in children (upper panels).

the findings in the healthy children, the lower limit of normal for buccal mucosal cells would be 15 ng/mg protein. If 15 ng/mg protein is used as the lower limit of normal for buccal cell tocopherol, approximately 37% of healthy newborn infants had values below the lower limit. The buccal cell tocopherol data did not confirm previous clinical impressions based on RBC tocopherol levels and tocopherol/lipid ratios (9, 10).

2. Changes in tocopherol concentrations in buccal mucosal cells after administration of tocopherol, compared with RBC and plasma concentrations

After the oral administration of 600 mg of RRR-alpha-tocopherol/day for 3 months to young adults, the tocopherol concentrations in buccal mucosal cells, RBCs, and plasma respectively increased to 3.5 times, 2.2 times, and 2.8 times the
Table 2. Changes in tocopherol concentrations in buccal cells, RBCs, and plasma in healthy young adults after daily administration of 600mg of RRR-alpha-tocopherol for three months.

<table>
<thead>
<tr>
<th>Time of administration</th>
<th>Buccal cells (ng/mg protein)</th>
<th>RBCs (µg/100 ml packed cells)</th>
<th>Plasma (µg/100 ml)</th>
<th>Tocopherol/lipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>49.2±18.5</td>
<td>226±19</td>
<td>772±165</td>
<td>1.76±0.32</td>
</tr>
<tr>
<td>1 month</td>
<td>129.9±113.3</td>
<td>517±83</td>
<td>2,279±513</td>
<td>4.79±0.98</td>
</tr>
<tr>
<td>2 months</td>
<td>102.6±22.1</td>
<td>470±88</td>
<td>2,113±552</td>
<td>4.38±0.91</td>
</tr>
<tr>
<td>3 months</td>
<td>173.4±53.2</td>
<td>498±90</td>
<td>2,176±499</td>
<td>4.76±0.89</td>
</tr>
<tr>
<td>1 month after cessation</td>
<td>116.4±86.2</td>
<td>220±20</td>
<td>927±125</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are the M±SD (n=18).

original values (Table 2). The increase of the tocopherol/lipid ratio was the same as that in the plasma tocopherol level, because no changes occurred in plasma total lipid levels during the study period. Plasma and RBC tocopherol levels reached a maximum after one month of supplementation, and thereafter continued at plateau levels, while buccal cell tocopherol continuously increased during the administration period.

The correlation between tocopherol concentrations in buccal mucosal cells and other samples, and between the tocopherol/lipid ratios and the other samples are shown on all the measurements before and after administration in Fig. 2. As shown in Fig. 2, buccal mucosal cell tocopherol levels showed a relatively poor correlation with the RBC and plasma tocopherol levels and the tocopherol/lipid ratios, while the tocopherol/lipid ratio was closely correlated to plasma and RBC tocopherol levels. This indicated that changes in the tocopherol content of the buccal mucosal cells after administration were different from those in plasma and RBC tocopherol levels.

The time courses of changes in the tocopherol levels in buccal mucosal cells, RBC, and plasma after a single dose of 600mg of RRR-alpha-tocopherol were investigated, as shown in Fig. 3. RBC and plasma tocopherol concentrations reached maximum levels within 24h, while the maximum level in the buccal mucosal cells was only reached 4 to 6 days later. This finding indicates that transportation of tocopherol to buccal mucosal cells takes more time after absorption than it does for plasma and RBC. This could explain the poor correlation of buccal mucosal cell tocopherol levels to plasma and RBC tocopherol levels and the tocopherol/lipid ratios. Since buccal mucosal cells seem to approximate the situation of most body cells, measurement of the tocopherol concentration in the buccal mucosal may be a better method for assessment of the vitamin E nutritional status of tissues.

3. Tocopherol concentrations in buccal mucosal cells in obese and hyperlipemic children

In our previous investigation of RBC tocopherol levels (12), the level in highly obese and hyperlipemic children has been reported to be lower than that in age-matched less obese and normolipemic children. In this study, we attempted to
Fig. 3. Changes of tocopherol concentrations in buccal cells, RBCs, and plasma after a single oral dose of 600 mg of RRR-alpha-tocopherol in healthy adults. Results are the mean ± SD for 5 young male adults.

confirm on the basis of buccal cell tocopherol changes whether obesity and hyperlipemia affected the tissue tocopherol concentration. In the obese children, tocopherol levels in plasma, RBC, and buccal cells did not differ between boys and girls (data not shown). As shown in Table 3, buccal cell tocopherol concentrations were significantly lower in very obese (obesity grade of 40% or more) children, when compared with those in non-obese children ($p < 0.01$). RBC tocopherol levels in this very obese group were also significantly lower than those in the non-obese group, indicating that the changes in RBC tocopherol content were consistent with the findings of our previous report (8). Plasma tocopherol levels were significantly higher in very obese children, probably due to their hyperlipemia, because there was no difference in tocopherol/lipid ratio between the groups. These obese children
Table 3. Tocopherol concentrations in buccal mucosal cells, RBCs, and plasma in obese children.

<table>
<thead>
<tr>
<th>Obesity grade (%)</th>
<th>N</th>
<th>Age (years)</th>
<th>Buccal cells (ng/mg protein)</th>
<th>RBCs (µg/100 ml packed cells)</th>
<th>Plasma (µg/100 ml)</th>
<th>Toc/ lipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 or less</td>
<td>27</td>
<td>9.5 ± 1.5</td>
<td>46.7 ± 25.9</td>
<td>206.3 ± 19.2</td>
<td>669.7 ± 176.1</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>20–29</td>
<td>19</td>
<td>9.5 ± 1.5</td>
<td>50.7 ± 34.7</td>
<td>208.8 ± 21.8</td>
<td>795.9 ± 190.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>30–39</td>
<td>19</td>
<td>9.4 ± 2.3</td>
<td>41.0 ± 29.6</td>
<td>207.6 ± 35.1</td>
<td>836.1 ± 200.5*</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>40 or more</td>
<td>17</td>
<td>9.5 ± 2.4</td>
<td>31.6 ± 10.3**</td>
<td>189.4 ± 23.9*</td>
<td>868.2 ± 154.8**</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

Values are the M±SD, **p < 0.01, *p < 0.05 (vs the 19% or less group).

Table 4. Tocopherol concentrations in buccal mucosal cells, RBCs, and plasma in hyperlipemic obese children, as compared with those in normolipemic obese children.

<table>
<thead>
<tr>
<th>Total lipids (mg/100 ml)</th>
<th>N</th>
<th>Age (years)</th>
<th>Obesity grade (%)</th>
<th>Buccal cells (ng/mg protein)</th>
<th>RBCs (µg/100 ml packed cells)</th>
<th>Plasma (µg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 500 mg</td>
<td>38</td>
<td>9.6 ± 1.6</td>
<td>36.9 ± 11.4</td>
<td>48.3 ± 28.0</td>
<td>209 ± 23</td>
<td>758 ± 147</td>
</tr>
<tr>
<td>500 mg or more</td>
<td>21</td>
<td>9.5 ± 2.1</td>
<td>39.8 ± 19.6*</td>
<td>33.7 ± 14.4*</td>
<td>195 ± 25*</td>
<td>948 ± 159**</td>
</tr>
</tbody>
</table>

Values are the M±SD, **p < 0.01, *p < 0.05.

were divided into two groups on the basis of plasma total lipids: one with a plasma lipid level of 500 mg/100 ml or more and the other with a level of less than 500 mg/100 ml. Age did not differ between the groups, while there was a slight difference in obesity grade. Plasma tocopherol levels were higher in hyperlipemia, as has previously been reported (1, 11–13), while buccal mucosal cell tocopherol levels were significantly lower in hyperlipemic obese children than in normolipemic obese children (p < 0.05, Table 4). The RBC tocopherol level was concomitantly lower in the hyperlipemic group than in the normolipemic group (p < 0.01), as reported in our previous paper (12). This finding appears to confirm the concept that increased lipid pools in the body, including adiposity and hyperlipemia, can act as a relatively nonexchangeable depot for tocopherol and can drastically affect its uptake by other tissues, possibly as a result of competition between the excess amounts of lipids in plasma and adipose tissue and the membrane lipids of other tissues (20, 21).

We have previously studied RBC and platelet tocopherol levels as an index of vitamin E status (8, 9, 12, 15). However, RBC and platelets are specialized cells without nuclei or low-density lipoprotein (LDL) receptors. Traber and Kayden (22) reported on the significance of LDL tocopherol in the delivery of tocopherol to tissue cells. In this light, we suggested that the direct measurement of tocopherol
concentrations in buccal mucosal cells, which contain LDL receptors, should provide information that is of clinical value, particularly in view of the low correlation between buccal mucosal cell tocopherol levels and those in blood components.

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