

Uptake of vitamin A in buccal mucosal cells after topical application of retinyl palmitate: a randomised, placebo-controlled and double-blind trial

U. Sobeck, A. Fischer and H. K. Biesalski*

University of Hohenheim, Department of Biological Chemistry and Nutrition, Fruwirthstr. 12, D-70593 Stuttgart, Germany

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Retinoids have been reported to produce regressions in metaplastic changes of the mucosal epithelium. In order to define the role of these micronutrients in the prevention of squamous metaplasia of the oral cavity, it is necessary to measure their uptake in target tissues such as the buccal mucosal epithelium. We demonstrated in a trial that retinyl palmitate applied topically via a toothpaste is taken up by buccal mucosal cells in young healthy volunteers. In the randomised, parallel-designed, placebo-controlled and double-blind trial, forty volunteers divided in two groups cleaned their teeth either with a placebo toothpaste or a retinyl palmitate-containing toothpaste (1 mg/g) for 56 d. Buccal mucosal cells samples were taken from the healthy volunteers during the retinyl palmitate application and the following wash-out phase to determine the concentration of retinyl palmitate and retinol by HPLC. Supplementary blood samples were taken from the volunteers on days 0 and 56 to investigate changes in plasma retinyl palmitate and retinol concentrations. Results from only thirty participants (sixteen placebo and fourteen treated subjects) were used in the statistical evaluation as the remaining sample results were spoiled by a technical defect during the HPLC analysis. A significant ($P < 0.05$) uptake of retinyl palmitate in buccal mucosal cells after 7 d and a significant ($P < 0.05$) increase of plasma retinol after 17 d was demonstrated in our present study. The uptake of retinyl palmitate and the following hydrolysis to retinol led to an enrichment of vitamin A in buccal mucosal cells.

Vitamin A: Retinyl palmitate: Buccal mucosa: Squamous epithelium: Oral care

Vitamin A and its compounds are essential micronutrients. Retinyl palmitate (RP) and its metabolites (retinal, retinol (ROH) and the most active metabolite, retinoic acid) play an important role in spermatogenesis, hearing, smelling, control of growth and epithelial differentiation, and in the visual cycle (retinal) (Gudas, 1994).

In the human body, dietary retinoids are taken up by the intestinal mucosa in the form of ROH. ROH is then re-esterified to RP, and released via the lymphatic system in the blood. It is further absorbed by the liver, and bound as ROH to the cellular ROH-binding protein or stored as RP in the stellate cells. When vitamin A is required by peripheral tissues it is re-mobilised by the liver. First, vitamin A is re-mobilised by hydrolysis, then it is bound to the ROH binding protein, released as a ROH-binding protein–transthyretin complex into the blood circulation, and is finally taken up by the peripheral tissue, depending on its need for vitamin A (Dawson, 2000).

Prolonged vitamin A deficiency, acute and chronic inflammation, and toxins, can result in a focal appearance of squamous cells in different mucous membranes, which

expand to replace normal epithelium (Hakansson & Ahlberg, 1985; Biesalski, 1996; McCullough *et al.* 1999). This leads to a lower epithelial barrier function and a higher risk of infection, as shown by Quadro *et al.* (2000) and Bloem *et al.* (1990). According to Biesalski & Stofft (1992) and Stofft *et al.* (1992), an increase in goblet cells and a decrease in ciliated cells can be detected in the respiratory tract during vitamin A deficiency. Guzman *et al.* (1996) showed *in vitro* that vitamin A controls the development and maintenance of mucociliary differentiation in the respiratory epithelium. Ponnampereuma *et al.* (1999) demonstrated that squamous metaplasia of vaginal epithelium is caused by a vitamin A-deficient diet in ovariectomised mice.

As the respiratory tract the buccal mucosa is exposed to chemical irritation and toxins and is at risk of developing metaplastic changes. In the study presented here, the uptake of topically applied RP in buccal mucosal cells is evaluated. This provides an opportunity to fill up RP stores, to circumvent the hepatic pathway and to increase the bioavailability of micronutrients in special target

tissues. It was found that topical application of RP resulted in a significant ($P < 0.05$) uptake of vitamin A in the buccal mucosa, and also in a physiological and controlled metabolism of RP to ROH.

Materials and methods

Concentration and purity of toothpaste

An RP-containing toothpaste (1 mg/g, Aronal[®] forte; GABA International, Therwil, Switzerland) or a placebo-toothpaste (Aronal[®] forte placebo; GABA International) was used in the morning during the trial. Elmex[®] toothpaste (GABA International) was used by all volunteers in the evening.

Study design

Forty healthy participants, twenty females and fifteen males, aged 19–33 years with BMI 18–24 kg/m² took part in the study. Excluding criteria were smoking, current dental surgery, illness of the pharynx or the cavity of the mouth, malabsorption, long-term medication, use of a toothpaste containing vitamin A the last 2 months before the study, pregnancy and metabolic diseases.

The duration of the study was 84 d. From days 0–56 the volunteers cleaned their teeth for exactly 3 min in the morning with a RP-containing or placebo toothpaste and in the evening with Elmex[®] (GABA International) by using the Aronal[®] öko dent toothbrush (GABA International) and a standardised quantity of toothpaste (1.7 g). Buccal mucosal cell samples were taken by the participants themselves during this phase on days 0, 3, 7, 10, 14, 17, 21, 28 and 56 with a surgical, soft toothbrush (TePe, Malmö, Sweden). During the wash-out phase, from days 56–84, the volunteers cleaned their teeth for exactly 3 min in the morning with the placebo formulation and in the evening with Elmex[®] (GABA International). Samples were taken on days 70 and 84. In addition, blood samples were taken on days 0 and 56 to investigate changes of plasma RP concentration and plasma ROH concentration.

Collection of buccal mucosal cells

Buccal mucosal cells were collected using a modified and improved method of Peng & Peng (1992). They were harvested by brushing a surgical soft toothbrush lightly across the inside of the cheek twenty times (one up–down stroke counted as one time) after rinsing the mouth thoroughly with drinking water. Immediately after brushing, the volunteers were asked to rinse their mouth with isotonic salt solution (9 g NaCl/l) to collect the cells.

Handling of samples and extraction

Buccal mucosal cells were collected in a plastic tube coated with 2,6-di-*tert*-butyl-4-methylphenole. The cells were washed three times with 10 ml PBS, centrifuged for 5 min at 1000 g (Sigma 3K15 centrifuge; Deisenhofen, Germany) and stored under Ar at –80°C.

After thawing, cells were cracked with liquid N₂ three times and a consecutive extraction with 200 µl ethanol and 1 ml *n*-hexane was performed twice. The upper hexane layer was carefully removed and dried under N₂.

Histochemical determination of buccal mucosal cells

Buccal mucosal cells were freshly harvested, stained by periodic acid–Schiff reaction and examined by light microscopy (Axioplan; Zeiss, Oberkochen, Germany). Reagents were obtained from Fluka (Buchs, Switzerland). During the periodic acid–Schiff reaction, cells were washed in a descending series of alcohol, stained in a periodic acid solution, in Schiff's reagent solution and in Boehmer's haematoxylin solution (Fluka). In between, cells were washed in running tap water and then rinsed in distilled water. Finally, cells were washed in increasing alcohol series and mounted in DePex resin (Serva, Heidelberg, Germany).

Handling and extraction of blood samples

Blood samples were obtained from each volunteer in EDTA-Monovetten tubes (Sarstedt, Nuembrecht, Germany). Afterwards, two portions of 200 µl and one of 1.5 ml blood plasma were frozen in microtubes at –80°C.

The two 200 µl blood plasma samples were extracted twice with 200 µl ethanol and 1 ml *n*-hexane. The hexane phase was removed, dried under N₂ and then re-dissolved in 100 µl mobile phase (acetonitrile–methanol (340:35, v/v)).

HPLC

RP and ROH were determined with a validated HPLC isocratic method (Paolo *et al.* 1999). The chromatograph system used was equipped with a Varian 9012 pump, a ProStar 410 Varian automatic injector and a L 4250 wavelength UV-Vis detector (Varian, Darmstadt, Germany). A Nucleosil 120, reversed-phase C₁₈, 300 × 3 mm, particle size 3 µm column (Grom, Herrenberg, Germany) was used for chromatography.

As mobile phase, we used acetonitrile–methanol (340:35, v/v) delivered at a flow rate of 1.0 ml/min. Samples were reconstituted in 100 µl solvent. The UV-Vis detector (Varian) operated at 325 nm and 50 µl sample was injected into the column.

Determination of DNA in samples

After extraction, the vacuum-speed-dried cell samples were incubated for 24 h at 37°C with 200 µl acetaldehyde (1.6 ml/l), perchloric acid (200 ml/l) and 320 µl diphenylamine (40 ml/l) (Natarajan *et al.* 1994). The absorption was photometrically analysed at 750 nm (EL-340; Bio-Tek Instruments, Inc, Winooski, VT, USA).

Determination of cholesterol

In two 50 µl blood plasma samples, cholinesterase was added to divide the cholesteryl ester into cholesterol and fatty acids. In the next step, cholesterol oxidase converts

cholesterol into Δ^1 -cholesterol-3-*on* by releasing H_2O_2 , which is important for the reaction of phenol with 4-aminophenazone in the presence of peroxidase to a red chinonimin derivative. The concentration of the chinonimin was detected photometrically at a wavelength of 500 nm.

Dietary assessment

Food-frequency questionnaires were given to complete at home by the volunteers themselves. It was analysed by Ebis (FEP, Esslingen).

Statistics

Statistical evaluation of the pilot study was carried out using the U test (using GraphPad Prism software; GraphPad Inc., San Diego, CA, USA).

Values with a significance level of $P < 0.01$ were considered as highly significant, and values with $P < 0.05$ were considered as significant.

Ethical considerations

The volunteers provided informed consent and the study was performed according to the 1983 revision of the Helsinki Declaration. The study was approved by the Ethical Committee of the Landesärztekammer/Stuttgart, Germany.

Results

Figure 1 shows a histochemical determination of reddish-stained single cells and cell agglomerates, which contain large blue chromosomal structures. Cell samples harvested in our present trial consisted of flat and differentiated cells that are characteristic for the outermost layer of the multi-layered buccal mucosa.

In the present trial, a significant increase of RP and ROH were found in buccal mucosal cells (Figs 2 and 3). However, results from only thirty participants (sixteen placebo and fourteen treated subjects) were used in the statistical evaluation. The remaining sample results were spoiled by a technical defect during the HPLC analysis.

From day 0 (0.034 pmol/ μ g DNA) to day 3 (0.007 pmol/ μ g DNA) a small but not significant decrease of RP was found. The concentrations of RP in buccal mucosal cells on days 0 and 84 (0.038 pmol/ μ g DNA) were nearly identical. The uptakes of RP on days 7 (0.065 pmol/ μ g DNA), 10 (0.092 pmol/ μ g DNA), 14 (1.780 pmol/ μ g DNA), 17 (3.041 pmol/ μ g DNA), 21 (4.163 pmol/ μ g DNA) and 56 (2.191 pmol/ μ g DNA) were significant ($P < 0.05$) compared with the placebo group (Fig. 2). During the wash-out phase, on day 70 (0.032 pmol/ μ g DNA), no increase of RP could be detected compared with day 0.

The change in concentration of ROH in buccal mucosal cells over the course of the study is shown in Fig. 3. ROH

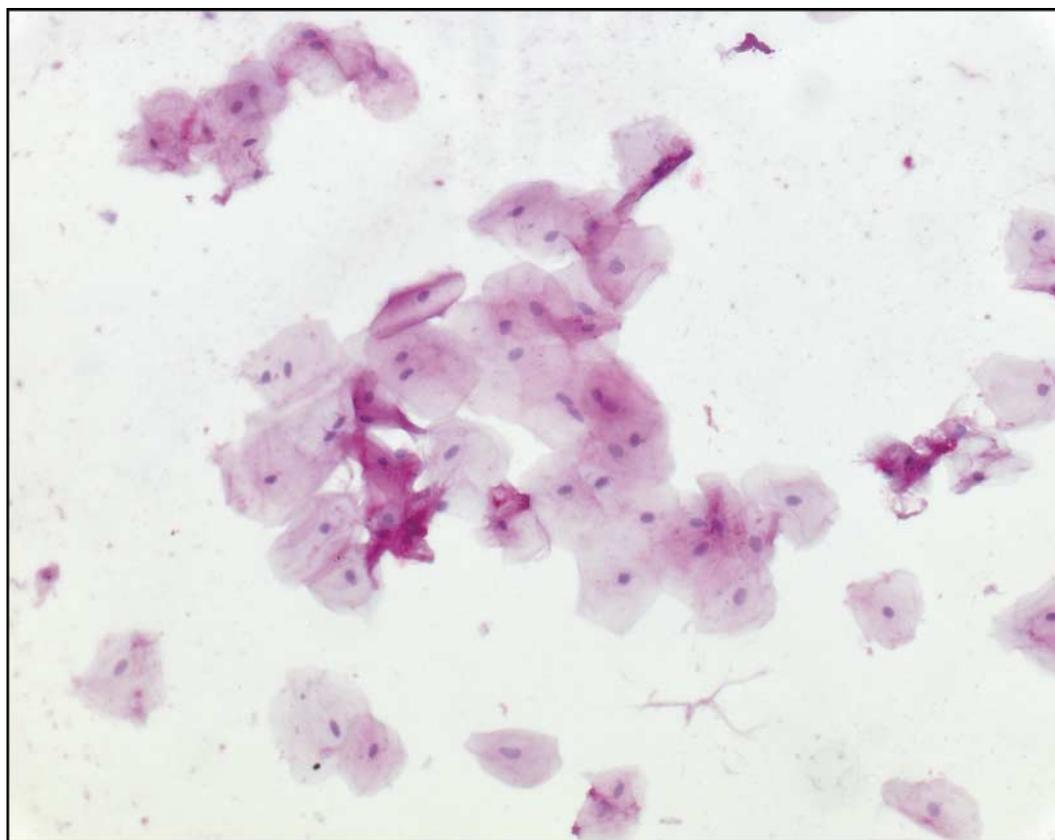


Fig. 1. Histochemical determination of buccal mucosal cells from young, healthy volunteers on day 0 before treatment. The figure shows reddish stained, flat-shaped cells with blue chromosomal structures. They are very characteristic for the outermost differentiated layer of the buccal mucosa. Cells were stained by periodic acid–Schiff reaction and examined by light microscopy. For details of procedures, see p. 70.

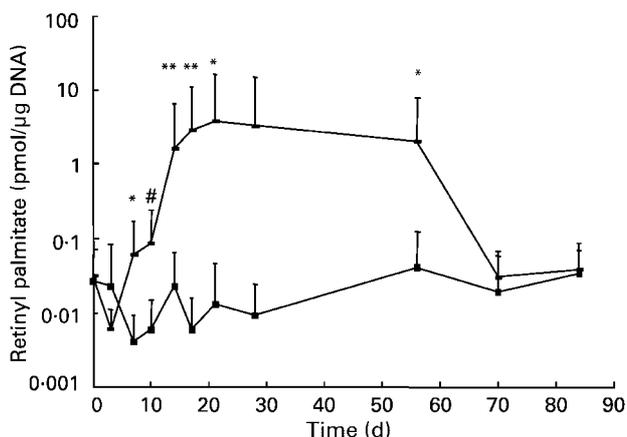


Fig. 2. Retinyl palmitate uptake in buccal mucosa cells (logarithmic scale) of subjects using a toothpaste containing 1 mg retinyl palmitate/g (*n* 14, 1.7 g/d, Aronal[®] forte; GABA International, Therwil, Switzerland) or a placebo toothpaste (*n* 16, Aronal[®] forte placebo; GABA International). Samples were taken on days 0, 3, 7, 10, 14, 17, 21, 28, 56, 70 and 84, and analysed by HPLC. —, Retinyl palmitate-treated group; ■, placebo group. For details of subjects, treatments and procedures, see p. 70. Values are means with standard deviations shown by vertical bars. Mean values were significantly different from those of the placebo group: **P*<0.05, ***P*<0.01.

concentrations showed a significant (*P*<0.05) increase on days 17 (0.181 pmol/μg DNA), 21 (0.268 pmol/μg DNA), 28 (0.208 pmol/μg DNA) and 56 (0.156 pmol/μg DNA) compared with the placebo group. A 10 d delay in the increase of ROH was detected, which was not found for RP. Regarding the wash-out phase, the levels of ROH decreased from day 56 to 70 (0.008 pmol/μg DNA). A small decrease was detected from day 70 to day 84 (0.003 pmol/μg DNA). Day 0 showed a concentration of 0.002 pmol/μg DNA.

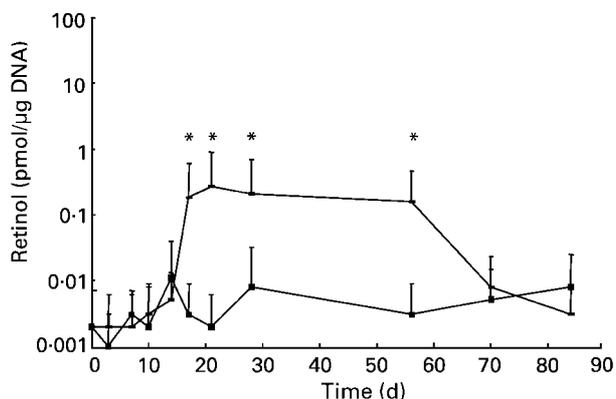


Fig. 3. Retinol uptake in buccal mucosal cells (logarithmic scale) of subjects using a toothpaste containing 1 mg retinyl palmitate/g (*n* 14, 1.7 g/d, Aronal[®] forte; GABA International, Therwil, Switzerland) or a placebo toothpaste (*n* 16, Aronal[®] forte placebo; GABA International). Samples were taken on days 0, 3, 7, 10, 14, 17, 21, 28, 56, 70 and 84, and analysed by HPLC. —, Retinyl palmitate-treated group; ■, placebo group. For details of subjects, treatments and procedures, see p. 70. Values are means with standard deviations shown by vertical bars. Mean values were significantly different from those of the placebo group: **P*<0.05.

To exclude effects of RP accumulation in the blood, samples were taken from the volunteers on days 0 and 56. No significant differences could be observed in the placebo and the treated group (Fig. 4). The values of RP as well as ROH showed low individual but high inter-individual differences, as found by Bitzen *et al.* (1994), who detected great differences in young healthy volunteers after an oral dose of RP.

The food-frequency questionnaire revealed that the volunteers had a high vitamin A consumption (placebo 2453 (SD 1187) μg/d, treated group 2313 (SD 1070) μg/d), similar to values found in a study conducted by Bingham *et al.* (1997). No significant differences were found between the treated group and the placebo group.

Discussion

The significant uptake of topically applied RP and its metabolism to ROH in peripheral tissue is of great importance, as retinoids play a pivotal role in growth and differentiation of epithelia (Massaro & Massaro, 1997; Biesalski & Stofft, 1992).

There are conflicting results concerning oral intake of vitamin A and the reversal of metaplastic alterations in mucosal tissue; however, topical application of RP opens new therapeutic possibilities for protecting and reversing such metaplastic alterations in different epithelial cells. Topically applied RP reaches the buccal mucosa in healthy volunteers. This leads to an enrichment of retinyl ester stores within the epithelium, independent of blood circulation (Biesalski, 1996). The metabolic pathway of vitamin A up to the conversion to retinoic acid, its most active metabolite, is strictly controlled by enzymes and binding proteins that provide a regulated shuttling of retinoids and shelter the retinoids from non-specific reactions (Napoli, 1999). Following cellular uptake RP is stored and metabolised, depending on the particular needs of normal and healthy cells, and only small amounts of RP

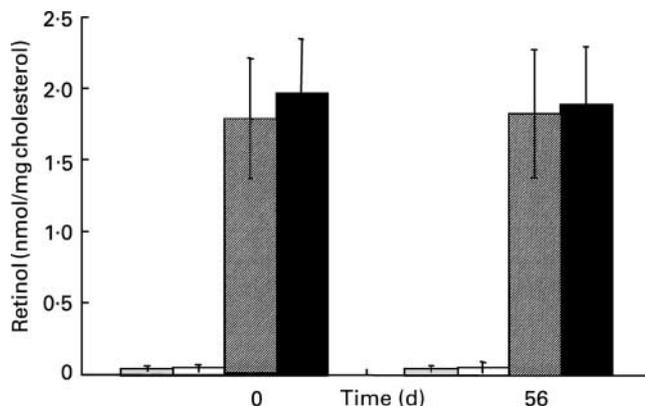


Fig. 4. Plasma retinyl palmitate (RP) and retinol (ROH) in subjects using a toothpaste containing 1 mg RP/g (*n* 14, 1.7 g/d, Aronal[®] forte; GABA International, Therwil, Switzerland) or a placebo toothpaste (*n* 16, Aronal[®] forte placebo; GABA International). Samples taken on day 0 and 56 were analysed by HPLC. ■, plasma RP in RP-treated group; □, plasma RP in placebo group; ▨, plasma ROH in RP-treated group; ■, plasma ROH in placebo group. For details of subjects, treatments and procedures, see p. 70. Values are means with standard deviations shown by vertical bars.

are hydrolysed to ROH. That finding is supported by a pilot study that showed a small, non-significant hydrolysis of ROH after topical application of RP in buccal mucosal cells (Sobeck *et al.* 2002). In cases of a cellular deficiency, topical RP application will result in typical cellular responses. Recently published studies propose that in cases of cellular vitamin A deficiency and metaplastic alterations, application of topical RP leads to a reversal of squamous alterations (Biesalski *et al.* 2001; Kohlhaufel *et al.* 2002). However, in cells with sufficient vitamin A supply, uptake of RP will not acutely result in either biochemical or physiological responses.

We applied a low dose of RP (5000 IU), which resulted in a significant increase of RP in the mucosa. Plasma RP and ROH did not increase after long-term application of RP for 56 d. Plasma ROH is homeostatically regulated, and as a consequence, ROH never increases, even when high doses are given. It is possible that there is a very small increase of plasma RP as a cause of unintentional swallowing of the toothpaste directly after application. However, RP in plasma (chylomicrons) only increases significantly following an intake of 10 000 IU, as shown by Willett *et al.* (1984) and Bitzen *et al.* (1994). In our present trial, the estimated uptake of RP in the buccal mucosal cells was <0.1% of the applied dose. As demonstrated in our present study, an intracellular pool of RP is established in cells after a treatment of 14 d. Therefore, topical application ensures a supply with vitamin A of mucosal epithelia independent from the delivery via the bloodstream. In vitamin A-deficient metaplastic epithelia it reaches peripheral tissues, as demonstrated by the reversibility of metaplasia and dysplasia of human respiratory epithelia after inhalation of vitamin A (Kohlhaufel *et al.* 2002). Metaplasia of rat vaginal epithelia was likewise shown to be reversed after topical application of RP (Biesalski *et al.* 2001). Further, it was proposed that in the nasal epithelium during rhinitis sicca, vitamin A is beneficial in maintaining and protecting a healthy epithelium (Breuninger & Khan, 1960). In addition, in cases of metabolic diseases (cystic fibrosis, malabsorption), topical application might be an important new therapeutic strategy to supply mucosal epithelia sufficiently with micronutrients. Its clinical relevance has to be investigated in ongoing trials.

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