CHAPTER 5

Nutritargeting

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Contents

Abstract

The term "nutritargeting" in analogy to the term "drug targeting" means targeting nutrients to specific "target" tissues.

What is the rationale for this idea?

Some tissues obviously are able to accumulate micronutrients selectively and to use them predominantly for specific functions. It has, for instance, been known for a long time that the accumulation of β-carotene in the skin does not only provide a "golden-yellow" color but considerable antioxidative protection as well. Yet

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β-carotene is only one of many antioxidants, which can be detected in the skin. Other carotenoids, for example, lutein and zeaxanthine, are preferentially found in the macula lutea, the so-called yellow spot in the eye. Here, carotenoids are subject to a metabolism typical for that tissue, which cannot be found in other tissues (e.g., formation of meso-zeaxanthine). In addition, they can specifically be absorbed into the macula. In the macula, they protect the retinal pigment epithelial cells against oxidative damage from UV light. Indeed, these two carotenoids can be protective against age-dependent macula degeneration.

Another example is the tissues that are particularly rich in vitamin C, for example, the cortex of the suprarenal gland or the lens: here, vitamin C fulfills both antioxidative functions and metabolic ones as it helps in the formation of collagen structures. Approximately 40% of the body's ascorbate is stored in skeletal muscle because this tissue is relatively abundant and its cellular concentration is tenfold higher than the plasma level. Similarly, the intracellular ascorbate concentration in the brain (3 mM) greatly exceeds the level in the extracellular fluid (200–400 μM). The majority of ascorbate is stored in the astroglial cells that are capable of reducing great quantities of DHAA to ascorbate, which then becomes available for release back into the extracellular fluid.

Thus, the accumulation of vitamins respectively micronutrients in single tissues is not limited to a pure storage process like the storage of vitamin A in the liver, but is often connected with important and tissue-specific metabolic functions.

When single micronutrients are applied for prevention or even intervention in diseases of organs or tissues, they are usually administered in higher doses for a longer period of time. The hope is to accumulate it this way sufficiently in the tissue and to thus be able to ensure the therapeutic success. This procedure, however, leads to a "flooding" of the whole organism with micronutrients and their potential enrichment in tissues which would usually not accumulate the respective micronutrient. Thus, unexpected side effects may occur.

An attractive solution to these problems in the future could be to wrap up or apply micronutrients in such a way that they can selectively reach the targeted tissue. For this approach, called "drug targeting" by pharmacologists, one could introduce the analogous expression "nutritargeting" with respect to micronutrients. For such a nutritargeting there are already a lot of examples and developments which show that it is possible to accumulate micronutrients in target tissues while simultaneously circumventing or protecting other tissues.

A substantial requirement for the development of "carriers" for nutritargeting is the availability of procedures or specific carriers, which allow the selected nutrients to bypass the main barriers that

are encountered when, for example, circumventing the enteral route in the targeting process. The entrance areas for such a targeting are the nasal mucosa, the oral mucosa, the cornea, the skin, or the lung. In the case of enteral application of proteins, the packaging has to resist gastric digestion and the body must be able to absorb the particles through the intestinal mucosa without hydrolyzing the proteins in order for them to reach the systemic circulation.

Another field in which nutritargeting may play an important role is the diseases where either systemic absorption is not possible (e.g., malabsorption/maldigestion) or where local deficits occur, which may not or only inadequately be supplied by systemic application.

I. NUTRITARGETING FOR SELECTIVE ACCUMULATION

A. Vitamin A

1. Rationale to use a topical (targeted) vitamin A supply

Vitamin A is essential for growth and development of cells and tissues. In its active form, retinoic acid (RA), it controls the regular differentiation as a ligand for retinoic acid receptors (RAR, RXR) and is involved in the integration (gap junction formation) of cell formations (Biesalski, 1996; Biesalski *et al.*, 1999). Vitamin A plays a substantial role, especially in the respiratory epithelium and the lung. During moderate vitamin A deficiency, the incidence for diseases of the respiratory tract is considerably increased and repeated respiratory infections can be influenced therapeutically by a moderate vitamin A supplementation (Biesalski *et al.*, 2001; Greenberg *et al.*, 1997; John *et al.*, 1997).

2. Significance of vitamin A for structure and function of the mature lung

A major target tissue for vitamin A is the respiratory tract and the bronchial epithelium. During a marginal vitamin A deficiency, prior to systemic effects, the sensitivity of the epithelium is increased due to a focal loss of ciliae and an increase of goblet cells (Stofft *et al.*, 1992a,b; Figs. 5.1 and 5.2). Similar morphological changes can be detected in heavy smokers with chronic obstructive pulmonary diseases (COPD), who show areas of local vitamin A deficiency but normal plasma levels (Auerbach *et al.*, 1979). Smoking and toxins [e.g., benzo(a)pyrene (BaP), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)] can cause a reduction in retinyl palmitate (RP) pools, the main storage reservoir of vitamin A in cells (Biesalski and Stofft, 1992) and at least in a local vitamin A deficiency.

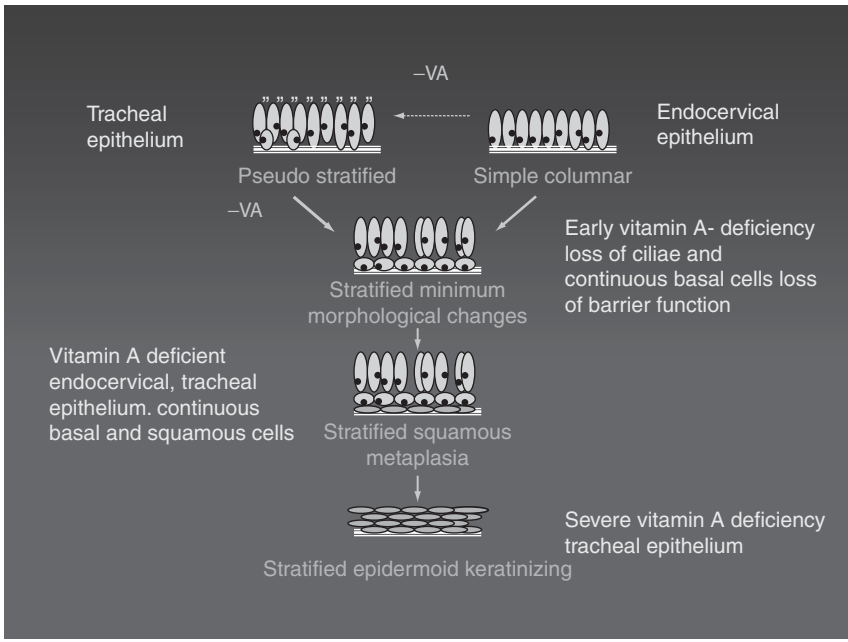


FIGURE 5.1 Influence of vitamin A status on epithelial phenotypes.

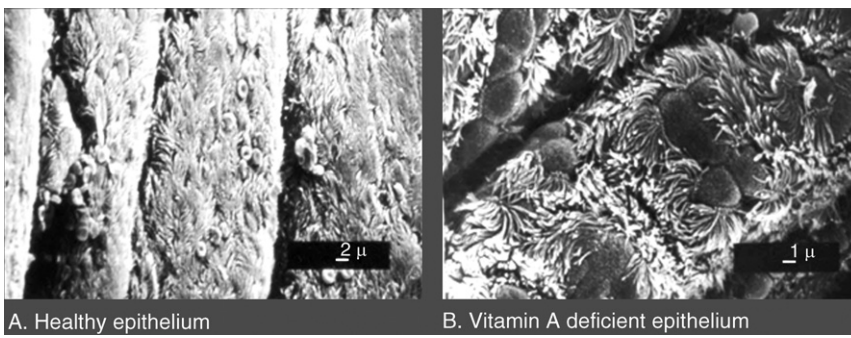


FIGURE 5.2 Morphological changes of the bronchial epithelium of the respiratory tract during vitamin A deficiency.

It has been reported that toxins (BaP, TCDD) are able to disrupt the normal vitamin A metabolism and interact with vitamin A metabolizing enzymes (LRAT, REH) in different tissues (Hanberg *et al.*, 1998; Nilsson *et al.*, 2000). In lungs, intestine, adrenals, and liver, it has been well established that BaP and TCDD affect these tissue stores of vitamin A and cause decreased levels of vitamin A (Biesalski and Stofft, 1992; Nilsson *et al.*, 2000).

Additionally, a further study described an increased catabolism and mobilization of vitamin A in the whole body (Kelley *et al.*, 1998).

On the basis of a few reports, it is assumed that a ''local'' vitamin A deficiency exists in meta- and dysplastic areas. Measurements of vitamin A concentrations in metaplastic areas of the respiratory epithelium and the cervix epithelium actually proved that vitamin A in comparison to the surrounding tissues was not found (Biesalski, 1996). Clearly one cannot say what is cause and effect. Studies carried out by Edes *et al.* (1991) confirm an induction of a vitamin A deficit. These studies showed that a depletion of vitamin A ester stores is caused by toxins, present in cigarette smoke (predominantly polyhalogenated compounds), in different tissues.

An essential importance for the development of obstructive respiratory diseases, within the scope of cancer mortality of smokers, was indicated by epidemiological studies. It was shown that the relative risk for smokers, with obstructive ventilation parameters [FEV 1% $<$ 60 (Melvyn *et al.*, 1987), respectively 70] (Skillud *et al.*, 1987), to be affected by lung cancer, is significantly higher than that of comparative groups with normal lung-function parameters.

A survey about the dietary habits within the scope of the ''National Health and Nutritional Examination Survey'' showed that an inverse correlation (Morabia *et al.*, 1989) exists between COPD and vitamin A supply as the only one of 12 examined dietary components. If a diminished supply of vitamin A increases the appearance of obstructive respiratory diseases, a marginal or local vitamin A deficit could be responsible for the observed changes of the respiratory mucosa. Such a deficit results in a loss of cilia, an increase of secreting cells and finally the formation of squamous metaplasia (Biesalski *et al.*, 1985; Chytil, 1985; Shah and Rajalekshmi, 1984).

Such changes (decrease of ciliated cells with simultaneous increase of the secretion) are noted for smokers (Gouveia *et al.*, 1982; Mathé *et al.*, 1983) and cause a reduction of the mucociliary clearance. This reduction of the mucociliary clearance, associated with an increased adsorption of the respiratory syncytial virus (RSV) (Donelly, 1996), could also explain the extraordinarily high morbidity and mortality for respiratory infections of children with vitamin A deficiency in developing countries (Sommer, 1993).

There is good evidence from experimental studies that the alteration of the respiratory mucosa, caused by the vitamin A deficiency, can be redifferentiated into its functional original epithelium, *in vivo* as well as *in vitro*, following vitamin A supply (Biesalski *et al.*, 1985; McDowell *et al.*, 1984a,b, 1987a,b; Rutten *et al.*, 1988a,b). Squamous metaplasia of the bronchial mucosa, which occurs in smokers in spite of a sufficient supply with vitamin A as an effect of inhalative noxae, could also be reversed through systemic application of high retinoid concentrations *in vitro* (Lasnitzki and Bollag, 1982, 1987) and in humans *in vivo* (Gouveia *et al.*, 1982; Mathé *et al.*, 1983).

The assumption of ''local'' vitamin A deficits as a basis for the inhalation approach is supported by studies, which showed that especially polyhalogenated compounds (e.g., TCDD) cause a local vitamin A depletion (Hakansson and Ahlborg, 1985; Thunberg and Hakansson, 1983; Thunberg *et al.*, 1980), which again contributes to the development of metaplastic- and possibly to dysplastic changes (Chopra and Joiakim, 1991). Thus, metaplastic changes are reversible by ''topic'' application (*in vitro*) of vitamin A (retinoic ester and RA). Consequently, a topical (inhalative) *in vivo* treatment of metaplasia of the respiratory tract could represent an efficient measure. In contrast, since RA is absorbed uncontrolled into the cells and a regulation of the cytoplasmatic retinoic acid-binding protein (CRABP) formation does not exist in that case, an application of inhalative RA is toxicologically more questionable.

By application of an inhalable vitamin A ester, an accumulation of the target cells can be achieved by much lower than the toxicological concentrations. In these target cells, the retinyl esters, after controlled hydrolysis, are released as retinol. Under the same controlled and consequently physiological conditions, retinol is re-esterified to the active metabolite RA. Consequently, the produced amount of retinol is adjusted to the respective amount of cytoplasmatic retinol-binding protein (CRBP) and along with it a corresponding amount of CRABP is expressed. Though many experiments *in vitro* as well as *in vivo* showed the effectiveness of RA on the reversibility of squamous epithelial metaplasia (see above), an inhalation of RA would hardly be justified because cellular regulation mechanisms are circumvented which is not the case for retinyl esters.

3. Treatment of squamous metaplasia with inhalation of vitamin A

Lung cancer is an extremely aggressive neoplasia, with the majority of admitted patients already showing metastatic dissemination. The best conventional treatment is a complete resection of local manifestation of bronchial carcinoma. However, only 40–50% of patients currently survive for >5 years after the surgery (Younes *et al.*, 1999). Primary prevention particularly eliminating exposure to tobacco, which is by far the most frequently encountered bronchial carcinogen, has failed to bring lung cancer under control. Thus, in the light of the poor prognosis of most patients diagnosed with lung cancer, alternatives to control the metaplasia-carcinoma-sequence are necessary. One approach is chemoprevention with vitamin A, which aims to arrest or reverse premalignant cells during their progression to overt malignancy (Sporn *et al.*, 1976). Progressive changes in the bronchial epithelium, that is, from squamous metaplasia to dysplasia and possibly carcinoma, have been proposed (Auerbach *et al.*, 1979; Boers *et al.*, 1996; Saccomano *et al.*, 1974). Squamous metaplasia of the respiratory mucosa occurs as a result of vitamin A deficiency (Jetten and Smits, 1985; Stofft *et al.*, 1992a,b). Vitamin A is the

generic term that describes an array of compounds (retinoids) that have the biologic activity of retinol. Retinoids are essential for embryonic development, cellular growth, and differentiation of different tissues including the tracheobronchial mucosa (Chytil, 1992; Hinds *et al.*, 1997; Stofft *et al.*, 1992a,b). Retinoids, including RA, retinol, and retinyl esters, can reverse squamous metaplastic changes of the respiratory epithelium as a result of exposure to metaplasia-inducing toxins *in vitro* and in animal experiments (Denning and Verma, 1994; Inayama *et al.*, 1996; McDowell *et al.*, 1984a,b). However, promising results of chemoprevention from application of high oral retinoid doses in former animal studies and human trials could not be confirmed by Lee *et al.* in a large trial (Gouveia *et al.*, 1982; Lee *et al.*, 1994; Mathé *et al.*, 1983; Nettesheim and Griesemer, 1978). This may be due to a lower bioavailability of oral retinoids for the target cells in the bronchial epithelium compared to *in vitro* studies, where vitamin A was topically applied (Biesalski, 1996). Further, inhalation of vitamin A (retinyl ester) has recently been reported to be an effective approach to supply the respiratory mucosa with vitamin A (Biesalski *et al.*, 1999). Retinyl esters are taken up by the respiratory mucosa and stored to serve as a plasma-independent source of vitamin A. Vitamin A inhalation avoids problems with absorption from the gastrointestinal tract, control of hepatic release, and cellular uptake, respectively. Thus, both the recent report of successful vitamin A supplementation in vitamin A-deficient children by inhalation of retinyl esters and the promising results of the new screening method (autofluorescence bronchoscopy) to detect intraepithelial premalignant lung lesions precisely (Biesalski *et al.*, 1999; Häussinger *et al.*, 1999, 2000; Lam *et al.*, 1998) provoked renewed interest in chemoprevention by inhaled retinoids.

At present no biopsy-proven data are available about the impact of inhaled aerosolized analogs of vitamin A on preneoplastic tracheobronchial lesions. The aim of a recent observational was twofold: first, to assess the feasibility RP inhalation and second, to investigate the response of epithelial changes (squamous metaplasia and dysplasia) to an inhaled RP aerosol in current smokers and ex-smokers (Kohlhäufl *et al.*, 2001).

Nineteen subjects with biopsy-proven diagnosis of metaplasia or dysplasia of the bronchial mucosa were recruited for the study at the 300 bed — Asklepios Center for Respiratory Medicine and Thoracic Surgery, Munich-Gauting, which is a specialized secondary care referral center performing 3000 bronchoscopies per year. Premalignant lesions in the bronchial epithelium were identified by using white light (WL)- and autofluorescence (AF)- bronchoscopy. On the second bronchoscopy, biopsies were taken at the identical areas. These areas were identified by the protocol of the initial bronchoscopy. In addition, the exact location was guided by AF bronchoscopy, which shows a reduction of AF intensity in areas of previous biopsies as well as in areas with premalignant lesions.

Individuals who met the eligibility requirements underwent a screening biopsy using AF bronchoscopy as an adjunct to WL bronchoscopy after a 3-month therapy with RP with two inhalations per day (each around 3.000 IU). In all patients, three endobronchial biopsies were taken again from three preselected sites: the bifuration of the right upper lobe, left mainstem bronchus, and the right segment 6. Further, AF bronchoscopy guided the sampling site for any additional suspicious lesions. If additional suspicious lesions were observed by AF bronchoscopy, they were biopsied.

Baseline plasma levels of RP of the study group increased but did not differ significantly from plasma levels after the 3-month inhalation trial (54.4 ± 32.6 nmol/liter vs 99.7 ± 68.2 nmol/liter; $p = 0.12$).

This preliminary study supports the feasibility of vitamin A application by aerosolized retinyl esters and showed a significant response of premalignant lesions of the bronchial epithelium. Complete reversal of metaplasia or dysplasia was noted in 44% of the biopsies. Partial remission of bronchial lesions was noted in 12% of the biopsies. The overall response rate (remission and partial remission) was 56% (confidence interval 0.30–0.79; $p < 0.05$). The large range of the confidence interval can be explained by the small number of participants in this preliminary study. The lack of a control group in this pilot study suggests caution in immediate application of these results. Since all smokers continued to smoke during the study, the epithelium was continuously exposed to carcinogenic, cocarcinogenic, or promoting substances. Thus, it seems unlikely that in this high-risk group the preneoplastic epithelial lesions can be reversed spontaneously. A recent prospective study reported no spontaneous remission of dysplastic areas in smokers for a 4-year period. Malignancy occurred in 25% of patients with grade I dysplasia, in 50% of patients with grade II dysplasia, and in 75% of patients with grade III dysplasia (Ponticello *et al.*, 2000). Also, among subjects who continued to smoke the metaplasia index did not change over a 6-month period in a randomized placebo-controlled trial of oral vitamin A supplementation (Lee *et al.*, 1994). These results are supported by an earlier animal study (hamster model). These studies showed that in carcinogen-treated animals areas of metaplastic epithelium did not reverse, but became atypical and progressed to carcinoma *in situ*. In contrast, only those areas of metaplastic epithelium were reversible which were induced by ''pure'' inflammatory agents (McDowell *et al.*, 1978). It was also shown that heavy smokers who stop smoking need at least two additional years to recover a normal bronchial histology (Bertram and Rogers, 1981). However, at present bronchoscopic studies are not available, which provide long-term data concerning the spontaneous remission rates of these early epithelial lesions in smokers.

4. The rationale for vitamin A inhalation

In an intervention trial with oral supplementation of β-carotene (20 mg/day) and vitamin E (50 mg/day) for 6.5 years, the incidence and mortality of lung cancer increased in the β-carotene group (relative risk, RR: 1.18) (Albanes *et al.*, 1996). Similarly in another intervention study, lung cancer risk increased (RR 1.28) in subjects supplemented with 30-mg β-carotene and 25.000 IU vitamin A (RP) for a time period of 5 years (Goodman *et al.*, 2004). In animal (ferrets) experiments, it was documented that the combination of smoke exposure and β-carotene results in a strong downregulation of the RARβ and activation of AP-1, which may contribute to an increased lung cancer risk (Wang *et al.*, 1999). In contrast to these studies, in our study vitamin A inhalation was performed with RP in a low-dose and not with high-dose β-carotene. Furthermore, retinoids do not induce downregulation of RARβ (Li *et al.*, 1998). The inhalation of retinyl esters provides the cells with an intracellular available vitamin A source. Under normal dietary conditions, retinyl esters can be detected in different cells of the respiratory tract in high concentrations compared with other tissues except hepatic tissue (Biesalski *et al.*, 1990). It is assumed that retinyl esters either formed by esterification of intracellular retinol, bound to CRBP, or as demonstrated recently they were taken up as such from the bloodstream (Gerlach *et al.*, 1989; Napoli, 1996). From these intracellular pools of retinyl esters, retinol may be acquired following hydrolysis of the retinyl esters by means of the cholate-independent retinyl ester hydrolase which predominates in the lung (Biesalski *et al.*, 1990). Retinol bound to CRBP may then be further oxidized to RA which enters the nucleus bound to CRABP to interact with RARs and their target genes. So far all steps of RA formation as the biologically active compound are controlled to avoid critical accumulation of RA within the cells. The importance of the intracellular retinyl esters was recently shown in rat lung fibroblasts (McGowan *et al.*, 1997). The authors inhibited the hydrolysis of retinyl esters and consequently the formation of RA in lung fibroblasts. As a result of this inhibition, the expression and the steady state level of the tropoelastin mRNA were reduced. These findings suggest that intracellular retinyl esters are important sources for retinol and RA, respectively. During marginal vitamin A deficiency, the retinyl ester stores of respiratory epithelium become rapidly depleted, while plasma retinol levels are only slightly decreased (Biesalski and Weiser, 1989; Biesalski *et al.*, 1990). This depletion of retinyl ester stores in the respiratory mucosa results in a loss of ciliae and an increase in mucous-secreting cells, an event which could lead to an impairment of lung function (Biesalski and Stofft, 1992; Biesalski and Weiser, 1990; Stofft *et al.*, 1992a,b). It is also known that an impairment of the mucociliary clearance increases the susceptibility against respiratory infectious diseases frequently associated with marginal vitamin A deficiency (Sommer *et al.*, 1984). Depletion of the retinyl

ester stores of the respiratory mucosa results in the development of squamous metaplasia which completely reverses to a normal epithelium after addition of vitamin A (*in vitro*) or dietary intake (animal experiments) (Denning and Verma, 1994; Inayama *et al.*, 1996; McDowell *et al.*, 1984a,b). The reversibility of cigarette smoke-induced metaplastic changes after addition of retinyl esters *in vitro* clearly documents that application of retinyl esters from the luminal side is an efficient approach (Rutten *et al.*, 1988a,b). Substances found in cigarette smoke condensate (i.e., BaP, polyhalogenated compounds) deplete tissue stores of retinyl esters (Fiorella *et al.*, 1995; Hakansson and Ahlborg, 1985; Rutten *et al.*, 1988a,b; Thunberg *et al.*, 1980). Exposure to these substances could result in a local vitamin A deficiency, which probably may not be compensated by systemic retinol therapy, but by topical retinol inhalation therapy.

5. Toxicological considerations

By inhalative application of vitamin A, an accumulation of peripheral vitamin A stores is achieved. For the lung and the respiratory epithelium, concentrations in the range of 1–20 μg/g were obtained (Biesalski, 1990). Looking at quantitative concentrations in the respiratory epithelium and in the mixed epithelium of the nasal mucosa yielded an accumulation of vitamin A — after topical administration in different animal species — in the epithelium of the nose increased by factor 10–100 (in human of factor 5–20) compared to the concentrations of the respiratory mucosa (Lewis, 1973).

The therapy of atrophic rhinitis by means of vitamin A-containing nose drops showed that high-dose topical application of the vitamin leads to the restitution of metaplastically modified nasal mucosa, side effects, especially differentiation impairments, were not reported (Breuninger and Kahn, 1960; Duncan and Briggs, 1962).

6. The influence of an insufficient vitamin A supply for the postnatal development of the lung

A disease seen recurrently in connection with vitamin A supply is the bronchopulmonary dysplasia (BDP). The pathogenesis of BDP certainly depends on a multitude of factors. Some of the observed morphological changes do remind strongly of the appearances as observed in vitamin A deficiency of humans and animals. Particularly noted should be the focal loss of ciliated cells with keratinizing metaplasia and necrosis of the bronchial mucosa as well as the increase of mucous-secreting cells (Stahlman, 1984; Stofft *et al.*, 1992a,b).

Especially focal keratinizing metaplasia, as it may occur after a vitamin A deficiency, is strengthening the assumption of an impairment of the differentiation on the level of the gene expression. Since vitamin A regulates the expression of different cytokeratins and therefore influences

the terminal differentiation, it seems obvious to suppose common mechanisms. Consequently, the premature but especially the neonate are dependent on a sufficient supply with vitamin A, to ensure the regulation of the cellular differentiation of the respiratory epithelium and lung epithelium. The earlier a child is born before the due date, the lower are its serum retinol levels (Mupanemunda *et al.*, 1994). Since a further decrease of the serum retinol level and retinol-binding protein (RBP) level occurs postnatally, the plasma value at the time of birth is described as a critical factor regarding lung development.

Repeatedly it was shown that the serum retinol level and RBP level in prematures are significantly lower than that of neonates (Shah and Rajalekshmi, 1984). In the liver of prematures, significantly lower retinol levels can be found in comparison to neonates (Shensi *et al.*, 1985). Plasma values lower than 20 μg/dl are not rare in this case and they should be taken as an indicator of a relative vitamin A deficit. But a moderate vitamin A deficiency is not only a problem of countries with poor or inadequate food sources.

Recently we published data that even in countries with excellent food sources and availability, insufficient vitamin A supply will occur (Schulz *et al.*, 2007). The aim of this trial was to analyze vitamin A and β-carotene status and investigate the contribution of nutrition to vitamin A and β-carotene supply in mother–infant pairs of multiparous births or births within short birth rates. Twenty-nine volunteers aged between 21 and 36 years were evaluated for 48 hours after delivery. In order to establish overall supply, retinol and β-carotene were determined in maternal plasma, cord blood, and colostrum via HPLC analysis. A food frequency protocol was obtained from all participants. Regardless of the high-to-moderate socioeconomic background, 27.6% of participants showed plasma retinol levels below 1.4 μmol/liter, which can be taken as borderline deficiency. In addition, 46.4% showed retinol intake <66% of RDA and 50.0% did not consume liver at all, although liver contributes as a main source for preformed retinol. Despite a high total carotenoid intake of 6.9 ± 3.9 mg/day, 20.7% of mothers showed plasma levels <0.5 μmol/liter β-carotene.

Retinol and β-carotene levels were highly significant correlated between maternal plasma versus cord blood and colostrum. In addition, significantly lower levels were found in cord blood [31.2 ± 13% (retinol), 4.1 ± 1.4% (β-carotene)] compared with maternal plasma. Despite the fact that vitamin A- and β-carotene-rich food is generally available, in contrast to developing countries, risk groups for low vitamin A supply indeed exist in the western world.

Reduced plasma levels during the first developmental months have a considerable influence on the total development as well as on the susceptibility for infections of infants. With reduced retinol plasma levels,

repeated infections are more often described (Barreto *et al.*, 1994; Filteau *et al.*, 1993) and they are counted among the main complications of a poor vitamin A supply in developing countries. In addition, the serum vitamin A level during infectious diseases, particularly of the respiratory tract, continues to drop (Neuzil *et al.*, 1994). On the one hand, this can be explained with an increased metabolic demand and on the other hand with a renal elimination of retinol and of RBP during the process of acute infections (Stephensen *et al.*, 1994).

7. Possibilities of prevention and therapy

On the basis of the importance of vitamin A as described above, the question arises to what an extent a therapeutical intervention can occur, especially for imminent premature deliveries but also for prematures, to achieve a prevention ahead of developing diseases and/or immaturities of the lung. One solution could be the intravenous administration of vitamin A, but due to the infusion systems used so far, it appeared that vitamin A is almost completely absorbed to the polyethylene tubes, respectively, and is damaged by light (Zachman, 1989). A possible improvement for the availability consists of coating the infusion systems with foil to avoid a further reduction of the vitamin due to light. Since the solutions are not available in the market anymore, and on the other hand new parenteral vitamin A preparations are not available yet, the significance of supplying the mother with vitamin A before the delivery must be pointed out.

The existing results of two randomized double-blind controlled studies of prematures show that the supplementation with vitamin A in a study lead to a considerable reduction (55%) of the risk to be affected by BDP (Pearson *et al.*, 1992; Shensi *et al.*, 1985). Another study though did not observe any changes. In a third study, 12 prematures received vitamin A intravenously for a period of 28 days (400 IE/day), and during later development, vitamin A was also administered orally (1500 IE/day) [Italian Collaborative Group on Preterm Delivery (ICGPd), 1993]. In the process of the supplementation, a significant change of the initially reduced plasma- and RBP-values occurred. The latter is an indication for an actual vitamin A deficiency of prematures because an increase of retinol-RBP can only be seen if a vitamin A deficiency really exists (principle of the relative-dose-response-test). A direct effect of the plasma concentration on the development of BPD could not be determined. The authors are coming to the logical conclusion that the plasma level (after delivery) hardly reflects the supply of the lung with vitamin A (before delivery). It has to be considered that in this study, it was again documented that especially prematures obviously feature a relative vitamin A deficiency. Thus, the attention should be directed to their supply with vitamin A. On the other hand, the vitamin A supply of the premature for achieving adequate concentrations in the lung either seems not

sufficient or the availability of the vitamin for the corresponding cells of the lung is not guaranteed. An alternative solution could be the inhalative application of vitamin A. With this, the lung is directly attained and retinyl esters administered by inhalation can be absorbed into the cells and metabolized in a controlled way, as shown in different animal studies (Biesalski, 1996; Biesalski and Weiser, 1993).

It should be elucidated to what extent the ''topical'' application of retinyl esters on the respiratory epithelium, especially with BDP, can contribute to the replenishment of the lung stores and thus leading to the improvement of the clinical outcome.

These results show that retinyl esters in respiratory epithelium and in alveolar cells form a pool of vitamin A, which can be used physiologically by the tissue. The formation of retinol and at least RA from retinyl esters is strictly controlled. So far an unphysiological formation of RA and a subsequent toxicity seems not possible. Retinyl esters, however, are biochemically inert with respect to gene expression or vitamin A activity as long as they are not hydrolyzed. Consequently, the inhalative application, especially in cases of insufficient lung development, could represent a true alternative. The oral contribution is hardly successful because of the poor RBP synthesis of the liver and the lack of availability of a parenteral solution is currently not available.

8. Vitamin A inhalation for treatment of vitamin A deficiency

Vitamin A deficiency is worldwide one of the most prevalent nutrition-dependent deficiency diseases. It leads to changes of the respiratory epithelium, which result in repeated infections of the respiratory tract, the main cause of death in vitamin A-deficient children. The difficulty in supplying the respiratory epithelium with vitamin A is that the affected children frequently suffer as well from infections of the gastrointestinal tract with subsequent reduction of the absorption of fat-soluble vitamins. Nutritargeting can in these cases avoid the problems of malabsorption and ensure the micronutrient supply.

The effectiveness of various therapeutical modalities of supplemental vitamin A has been examined in numerous studies using tablets or capsules. On the basis of these studies, increased consumption of dietary vitamin A has been advocated (World Health Organization, 1984, 1992). In India and Indonesia, the provision of extra vitamin A resulted in considerable reduction of mortality (ca. 40%) in preschool children (Bhandari *et al.*; 1994; Humphrey *et al.*, 1996). While the efficacy of excessive oral doses over more than 8–12 weeks has been questioned, it is evident that an insufficient, low dose given once per week is apparently of little effect on morbidity or mortality (Ramakrishnan *et al.*, 1995a,b). Indeed, recurrent diarrheal episodes or the existence of malnutrition may explain the poor efficacy seen with oral supplementation with low doses (Ramakrishnan *et al.*, 1995a).

In contrast to oral supplementation, the present investigation aims to evaluate the efficacy of inhalation of the vitamin as an alternative route bypassing absorption and liver storage. The advantage of this approach might be that problems frequently seen during protein calorie malnutrition (PCM) due to impaired RBP synthesis with impaired vitamin A release from the liver (Rahmathullah *et al.*, 1991; Ramakrishnan *et al.*, 1995a) can be compensated. In addition the most sensitive target tissue, the lung, is directly supplied.

In a placebo-controlled randomized supplementation trial (approved by the ethic commission of Ethiopia) in the rural area (AZOZO) district of Gondar Ethiopia from 220 households, 161 children (2–5 years of age) were selected at random for the study; at a first visit to the local clinic, nutritional assessment, and stool examination (parasites or ova) were performed (Biesalski *et al.*, 1999). 141 children with parasites were treated with mebendazole. Heparin blood was obtained for assessment of vitamin A, RBP, and TTR (transthyretine) concentrations.

Twenty-five children selected at random received aerosol treatment with RP; 6000 vitamin A units per 2 weeks over 3 months being provided. Twenty-five further children served as controls receiving a placebo also aerosol delivered. The aerosol was administered through the mouth during breath inhalation with an adapter. No adverse effects or reactions were observed during inhalation and the children complied well with the treatment. Trained field workers performed the inhalation trials and blood sampling. In the study and control group, Heparin blood samples were collected before and at completion of the study for measurements of vitamin A, RBP, and TTR concentrations.

The mean initial serum retinol concentration derived from the 161 children was 0.74 ± 0.46 µmol/liter. Fourteen children (8.7%) exhibited a vitamin A deficiency defined by extremely low serum retinol concentration <0.35 µmol/liter and 78 children (48%) revealed marginal deficiency as indicated by low serum concentrations (<0.70 µmol/liter). Serum retinol concentration was not different in the study- and control group prior to inhalation (Fig. 5.3).

The serum retinol concentration increased considerably (1.43 ± 0.46 µmol/liter, $p < 0.001$) compared with the initial value (0.68 ± 0.31 µmol/liter) following supplementation (six inhalations, totally 36.000 IU) and compared with the control group (pre: 0.75 ± 0.42; $p < 0.05$, post: 0.79 ± 0.37). The RBP concentration was low prior to the treatment (pre: 0.93 ± 0.12 µmol/liter) and increased after inhalation (post: 1.68 ± 0.24 µmol/liter). The concentrations in controls (pre: 0.89 ± 0.14 µmol/liter; post: 0.9 ± 0.11) remained low. Supplemental vitamin A did not affect TTR concentrations in the treatment (pre: 157.2 ± 43.7 mg/liter vs post: 170.9 ± 35.1 mg/liter) or the control group (pre: 165.7 ± 36.1; post: 168.1 ± 28 mg/liter).

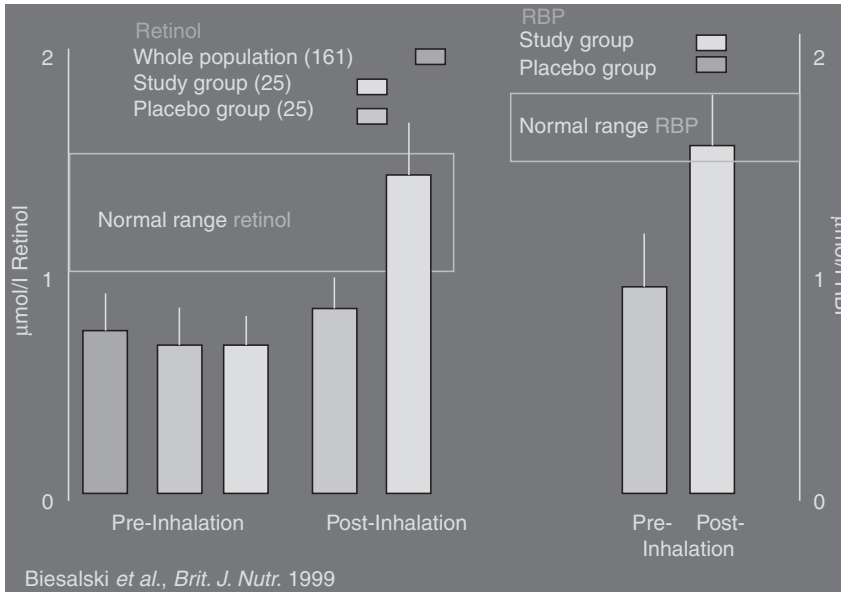


FIGURE 5.3 Inhalation of vitamin A improves vitamin A deficiency in Ethiopian children with severe fat malabsorption.

In this study, the depleted target tissues were supplied with retinyl ester implicating topical/systemic inhalation method, thereby first bypassing the GI-tract and liver. Indeed, following inhalation, the RP is likely to be carried to the liver where it is taken up, bound to RBP, and re-excreted into the circulation before it is utilized. Another assumption might be that the retinyl esters are taken up as such and may serve as a directly available cellular pool (Biesalski, 1996; Gerlach *et al.*, 1989). As shown, aerosol supported RP supplementation enhanced vitamin A and RBP concentrations, while no effect was observed in the release of TTR. Indeed, it is well known that TTR metabolism is regulated independently and that its changes in concentration changes are usually associated with alterations in nutritional status. Consequently, the considerable decreases in retinol and RBP concentrations in face of unchanged TTR levels indicates that these alterations are solely due to vitamin A deficiency or its supplementation. The fact that the release of RBP increased following inhalation strongly indicates that the observed vitamin A deficiency is primarily owning to low intake or malabsorption of vitamin A and not due to protein energy malnutrition (Blaner, 1989).

It is essential to examine whether the uptake of retinyl esters in the respiratory mucosa might be associated with an excess formation of free retinol or RA in the cells with the formation exceeding the binding

capacity of CRBP or CRABP. In experimental studies with inhalative application of retinol margarinate, the uptake of retinyl esters showed great variation in different sites of the lung tissue (Biesalski, 1996). Yet, the cellular concentration of retinol remained essentially unchanged. This indicates that the formation of retinol is strictly controlled despite high or low uptake of retinyl esters. In this connection, it is also necessary to emphasize that long-term topical administration of high vitamin A in liquids containing 15.000 IU/ml is an established therapy of atrophic rhinitis, rhinitis sicca, and further metaplastic changes of the nasal epithelium (Simm, 1980). The applications lead to the normalization of the epithelium and reappearance of a normal function without any reported side effects.

An impairment of the mucociliary clearance increases the susceptibility against respiratory infectious diseases frequently associated with marginal vitamin A deficiency (Sommer *et al.*, 1984). Interestingly vitamin A status following supplementation with 15-mg RP monthly for 2.5 months (Rahman *et al.*, 1996) was not improved in the presence of respiratory tract infections (Sommer *et al.*, 1986).

Inhalative application of vitamin A, as used in the present study, exerts direct and immediate effects on the epithelia of the upper respiratory tract. A further advantage of the inhalative route is a complete absorption of the retinyl ester, the rate being independent of the mucosal integrity of the gastrointestinal system. Indeed, the gut might be one of the less integrated organs as a consequence of vitamin A deficiency. Thus, inhalation of retinyl esters might be suitable for vitamin A therapy in the presence of malnutrition and diarrhea.

We found that supplementation of vitamin A in the form of an aerosol is an effective, safe, and routinely manageable method to enhance vitamin A and RBP concentrations. Consequently, this modality of treatment may serve as an alternative vitamin A therapy during chronic or acute episodes of malnutrition, malabsorption, or in case of insufficient compliance to other therapies and might be useful in respiratory diseases associated with vitamin A deficiency.

9. Topical supply of vitamin A to mucous membranes

Prolonged vitamin A deficiency as well as 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 (Biesalski, 1996; Hakansson and Ahlborg, 1985; McCullough *et al.*, 1999). This effect leads to a lower epithelial barrier function and a higher risk for infection shown by Quadro *et al.* (2000) and Bloem *et al.* (1990). According to Biesalski and Stofft (1992) and Stofft *et al.* (1992a,b), an increase in goblet and a decrease in ciliated cells can be detected in the respiratory tract during a 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. Ponnamperuma *et al.* (1999) demonstrated that squamous metaplasia of vaginal epithelium is a result of a vitamin A-deficient diet in ovariectomized mice.

In a recent study, we evaluated the uptake of topically applied RP in buccal mucosal cells (BMCs). This could be a way to circumvent the hepatic pathway and to increase the bioavailability of micronutrients in special target tissues (Sobeck *et al.*, 2003).

A 0.1% PR-containing toothpaste (Aronal® forte GABA *International* AG, Therwil, Switzerland) or a placebo toothpaste (Aronal® forte Placebo) was used in the morning for the clinical study. Elmex® toothpaste (GABA *International* AG) was used by all volunteers in the evening.

Forty healthy participants, 25 female and 15 males, aged 19–33 years with a body mass index between 18 and 24 kg/m^2 took part in the study. Excluding criteria were smoking, current dental surgery, illness of the pharynx or the cavity of the mouth, malabsorption, long-time medication, use of a toothpaste with vitamin A during the last 2 months prior to the study, pregnancy, and metabolic diseases.

The duration of the study was 84 days. From day 0 to 56, the volunteers cleaned their teeth exactly 3 min in the morning with an RP-containing or placebo toothpaste and in the evening with Elmex®, by using the Aronal® öko dent toothbrush and a standardized quantity of toothpaste (1.7 g). BMC samples were taken by the participants themselves during that phase on day 0, 3, 7, 10, 14, 17, 21, 28, and 56 with a surgical, soft toothbrush. During the wash-out phase from day 56 to 84, the volunteers cleaned their teeth exactly 3 min in the morning with the placebo formulation and in the evening with Elmex®. Samples were taken on day 70 and 84. Additionally, blood samples were taken from the on day 0 and 56 to exclude any side effects.

BMCs were collected using a recently modified and optimized version of a published method (Erhardt *et al.*, 2002). They were harvested by brushing a surgical soft toothbrush lightly across the inside of the cheek for 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 to collect the cells.

The period from day 0 (0.034 pmol/μg DNA) to day 3 (0.007 pmol/μg DNA) showed a short but not significant decrease of RP. The concentration of RP in BMCs on day 0 and day 84 (0.038 pmol/μg DNA) was nearly identical. The uptake of RP on day 7 (0.065 pmol/μg DNA), day 10 (0.092 pmol/μg DNA), day 14 (1.780 pmol/μg DNA), day 17 (3.041 pmol/μg DNA), day 21 (4.163 pmol/μg DNA), and day 56 (2.191 pmol/μg DNA) was significantly increased compared to the placebo group.

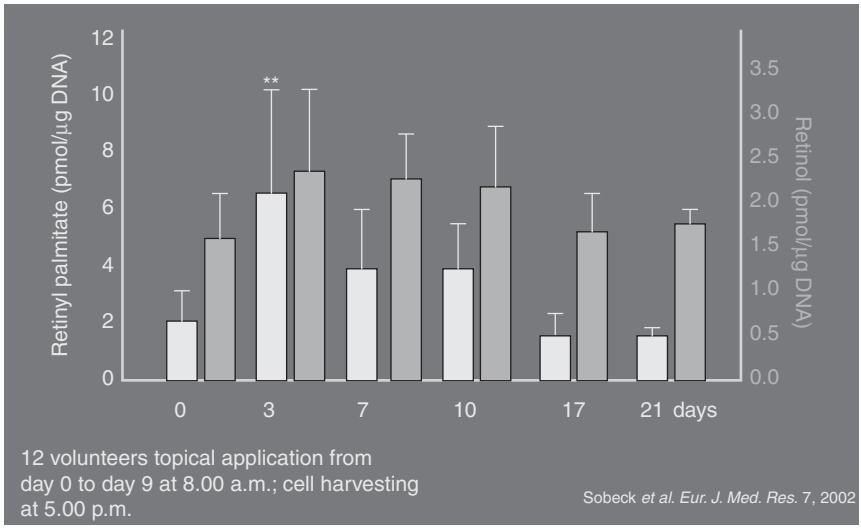


FIGURE 5.4 Uptake of RP (0.1%) and formation of retinol from dental gel into BMCs.

The course of retinol concentrations in BMCs is shown in Fig. 5.4. The values of retinol showed a significant increase between day 17 (0.181 pmol/µg DNA) and day 21 (0.268 pmol/µg DNA), 28 (0.208 pmol/µg DNA), and 56 (0.156 pmol/µg DNA) compared to the placebo group. A delay of 10 days in the increase of retinol could be detected compared to the course of RP. Regarding the wash-out phase, the levels of retinol decreased from day 56 to 0.003 pmol/µg DNA on day 84. Day 0 showed a concentration of 0.002 pmol/µg DNA.

To exclude effects of RP accumulation in the blood, samples were taken from the volunteers on day 0 and 56. No significant differences could be observed in the placebo and the treated group. Bitzen *et al.* (1994) detected high interindividual differences in RP as well as retinol levels in blood samples of young healthy volunteers after an oral dose of RP.

The significant uptake of topically applied RP and its metabolization to retinol in peripheral tissue is of high importance as retinoids play a pivotal role in growth and differentiation of mucous epithelia (Epstein and Gorsky, 1999; Massaro and Massaro, 1997).

We applied a low-dose RP (5000 IU), which resulted in a significant increase of RP in the mucosa but not in the plasma. Plasma retinol is homeostatically regulated and as a consequence retinol never increases even when high doses are given. In contrast RP in plasma (chylomicrons) increases significantly following an intake of greater than 10.000 IU shown by Willett *et al.* (1984) and Bitzen *et al.* (1994). These data also show that determination of micronutrients in buccal cells gives more valuable information on the individual status than plasma levels.

An absence of retinoids can lead to inflammation and metaplastic alterations such as gingivitis, shown by Gonzáles *et al.* (2001). Topically applied RP provides the buccal and gingival mucosa with a sufficient amount of vitamin A. We suggest that an enrichment with vitamin A may lead to prophylactic effects (e.g., a reduction of inflammatory diseases) or a therapeutic reversal of metaplastic alterations.

A prolonged Vitamin A-deficient diet results primarily in single changes of cellular terminal differentiation, changing into squamous metaplasia after complete depletion of the cellular storage. However, such cellular metaplastic changes also occur independently of the systemic supply, induced by local inflammation, pro-inflammatory cytokines, or chronic irritation (e.g., polychlorinated compounds), resulting finally in a local vitamin A deficiency. To reduce inflammation and subsequent irritation an adequate oral hygiene is an essential preventive action. An additional effect may be obtained by local application of vitamin A as persistent inflammation places an increased demand on this essential compound (Kanda *et al.*, 1990). Furthermore, a sufficient vitamin A level in mucosal tissues boosts the immune system to prevent oral inflammation of the gums.

10. Evaluation of the effects of topical vitamin A application on rat vaginal mucosa

Test substances applied to oral mucosa of animals are in part swallowed and therefore may act systemically. To prevent this, the tests were also carried out *in vivo* on the vaginal mucosa of rats. Studies on vaginal epithelia of ovariectomized (OVX) rats have shown that a vitamin A-free diet enhanced squamous metaplasia (Ponnamperuma *et al.*, 1999).

Since the oral mucosa is morphologically nearly identical to the vaginal one, this tissue is most suitable to test the efficacy and bioavailability of an oral care product. Furthermore, rats have the anatomical benefit of a completely separated vagina and urethra; the applied preparation therefore cannot be rinsed off by the urine. To exclude interference of the female sex hormones with the vitamin A state, ovariectomized animals were used as the appearance of squamous cells in the oestrous phase can morphologically not be distinguished from those of a vitamin A deficiency as shown by Ponnamperuma *et al.* (1999). Leukocytes were estimated as they indicate both, the diestrous phase and an adequate supply with vitamin A. In this experiment, we investigated the reversal of squamous metaplastic changes in rat vaginal mucosa as an indicator to test efficacy and bioavailability of topical vitamin A (Biesalski *et al.*, 2001).

The animals treated with 200, 400, and 800 IU A showed a healing effect of vitamin A on the cornified vaginal epithelium as early as 2 days after starting the experiment. In the smear of all concentrations, almost exclusively leukocytes — indicating a successful healing and mucosal regeneration — with only sporadic epithelial cells and squamous cells

were identified. The application of 200 IU of vitamin A resulted in a mean healing duration of 19.33 ± 1.21 (SD) days before completely cornified vaginal epithelium occurred again. The protective effect of 400 IU of vitamin A lasted 31.75 ± 0.96 (SD) days while 800 IU of vitamin A resulted in a healing duration of 43.0 ± 0.82 (SD) days. Fig. 5.5 demonstrates that the healing duration correlates with increasing concentrations of vitamin A palmitate. In the placebo-treated group, only a pure squamous cell stage was seen. As no change in the cell picture occurred up to four days after treatment, further observations were ceased.

The relative activity of vitamin A palmitate in Aronal forte toothpaste was compared to an experimental low-dose toothpaste and the formerly used dental gel as a standard. The animals treated with 384 and 768 IU of vitamin A dental gel, respectively, showed the healing effect as early as one day after the vaginal installations, leading to a mean healing duration of 31.3 ± 1.21 (SD) and 40.5 ± 0.56 (SD) days, respectively. The protective effect on the vaginal mucosa with 391 IU and 782 IU of vitamin A per animal of Aronal forte, respectively, lasted 33.3 ± 0.82 (SD) and 41.5 ± 1.05 (SD) days, respectively. With the low-dose toothpaste (305 IU of vitamin A), a mean protective effect of 29.3 ± 1.21 (SD) days was achieved. The administration of the double concentration per animal gave a clearly prolonged duration of 35.3 ± 1.63 (SD) days. The placebo-treated animals showed only squamous cells in the lavage fluid for up to 4 days after treatment; further observations were therefore stopped.

The statistical evaluation by means of the four-point parallel line assay resulted for the Aronal forte toothpaste in a significant higher activity of factor 1.11 (C.l. 1.03–1.19; $p = 0.05$) in comparison to the dental gel standard. The evaluation carried out on the quality of the experiment

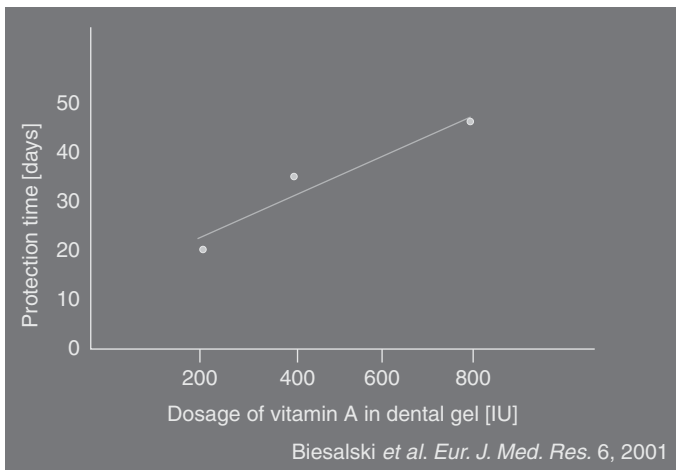


FIGURE 5.5 Epithelial protection time (until reappearance of squamous cells) following topical vitamin A application on vad-rat vaginal mucosa.

using the *t*-test gave a significantly different slope of the regression lines. This demonstrates a good dose-effect relationship. In the check for parallelism of the regression lines, the null hypothesis was confirmed. The relative efficacy of the experimental low-dose toothpaste was different by a factor of 0.92 (C.l. 0.82–1.02; $p = 0.05$; Finney, 1978). A good dose-effect relationship with significant slope of the regression lines was shown with the *t*-test. But in case of the parallelism, there was a significant difference.

In order to demonstrate pharmacological activity of vitamin A in Aronal forte toothpaste, treatment with 87.6 IU and 175 IU of vitamin A, respectively, and a retention time of 1 and 3 min were carried out for 11 days. The healing effect of vitamin A could be seen already 1 day after the first topical treatment with 1 or 3 min retention time prior to rinsing. The lavage fluid consisted of an equal mix of leukocytes, epithelial cells, and squamous cells, indicating the start of the healing process upon the cornified mucosa. As treatment progressed, a correlated increase in the proportion of leukocytes was observed, as sign of mucosal productivity. After the last application, cell differentiation was continued until pure squamous cells appeared. Protective effects of 7.60 ± 1.14 (SD) and 8.0 ± 0.82 (SD) days were registered with the lower and higher dose.

At a retention time of 3 min, the cell picture in the smear changed from a pure squamous type to a mixture of leucocytes and epithelial cells after the first day of treatment. With progression of the treatment, the leucocytes became the dominant cell type, corresponding to an increase in epithelia cells. After the cessation of the treatment on day 11, the examination of the smears was continued until pure squamous cells were found as a result of the vitamin A being used up. The protective effect of vitamin A was registered to be 7.80 ± 0.84 (SD) and 9.75 ± 0.96 (SD) days, respectively.

By means of the parameter-free, one-sided X-test, the protection in days after cessation of the vitamin A supply was statistically evaluated (Van der Waerden and Nievergelt, 1956). These results question whether an increase in amount and retention time of Aronal forte toothpaste results in an elongation of epithelial protection. With the short supply of vitamin A for 1 minute, no dose-dependent improvement was detected. However, with a retention time of 3 minutes, the higher dose correlated positively with the duration of vitamin A protection. The differences between the higher and lower dose were statistically significant ($p < 0.025$).

The control group was treated twice daily with 0.10 ml of placebo with rinsed out after 3 minutes. The squamous cell phase seen initially did not change with the treatment. After 4 days, the administration of placebo had to be discontinued as the general condition of the animals had deteriorated.

The conducted experiments confirm the hypothesis that topically applied vitamin A is taken up fast into deeper cell layers and exerts pharmacological activity on mucous membranes. For the first time, we evidently demonstrated that squamous epithelial changes induced by vitamin A deficiency of OVX-rats are totally reversed in to a fully healthy

status by topical application of RP in dental care products. Applying a total amount of 800 IU over 2 days exhibited healing and protective activity from total keratosis for 43.0 ± 0.82 days. Restricted retention time of 1 min and low concentration (87.6 IU vitamin A) twice daily still show healing effects 24 h after first application. The squamous cells were no longer predominant and, with progressive healing, the proportion of leukocytes and epithelial cells grew. After only 2 days of treatment, squamous metaplastic vaginal epithelium showed a full reversal of the epithelium into a healthy status, protected for another 7–11 days from full cornification after completed therapy. Higher concentrations (157 IU) and longer retention of 3 min lead to a statistically significant ($p = 0.025$) increase of the protection time. Furthermore, *in vivo* where a significant enrichment of RP from Aronal forte in human buccal mucosa was measured (Sobeck *et al.*, 2003). No more retinyl esters were detected ~17 days after completion of the treatment, indicating a washout during the cellular differentiation. We therefore conclude a fast uptake and long storage of vitamin A occurs in epithelial cell layers.

The obtained results confirm earlier findings where vitamin A-deficient rats were used to prove the uptake of retinyl esters into lung, liver, kidney, and plasma after inhalation thereof (Biesalski, 1996). However, long-term topical administration of high vitamin A concentrations is a well-established therapy in atrophic rhinitis, rhinitis sicca, and metaplastic changes in the nasal or ocular epithelium (Deshpande *et al.*, 1997; Simm, 1980). The application leads to the normalization of mucous membranes and reappearance of a normal function with no side effects.

Our findings are most important as vitamin A deficiency is believed to compromise mucosal immunity by altering the integrity of mucosal epithelia of the genitourinary tract (Semba, 1998) and most likely also the oral tract. Retinoids are important for the regulation and cell differentiation of epithelial layers. Their absence provokes inflammatory changes, cornification, and metaplasia and therefore weakens the natural barrier function of the mucosal membrane. In addition, the secretory IgA response to various pathogens is thought to be impaired (Aukrust *et al.*, 2000) and mucin production on the transcriptional level is disturbed (Guzman *et al.*, 1996). Mucin, however, plays an essential role as a first line of defense in the oral cavity. Topical vitamin A delivery on the oral or genital tract may in conclusion improve the endothelial barrier function and contribute to a lower risk for viral transmission or pathogenicity of microorganisms (Semba, 1998). Interestingly, we also observed an improved general health in animals treated with the dental care products while placebo-treated animals were only kept alive by oral vitamin A supply. Together with the findings of the experiments with Aronal forte, these results support the use of vitamin A by topical application onto mucous membranes as a basic supplement in malnourished people. A prerequisite though is high

availability and biological activity of vitamin A palmitate from the vehicle. Furthermore in contrast to studies with high oral intake of vitamin A, no toxic effects were seen. An intracellular pool of vitamin A independent from the bloodstream could be established by targeted treatment of vitamin A palmitate which might circumvent these toxic effects.

B. Vitamin E

Similarly, the inhalation of vitamin E could be an effective way to selectively supply the lung with this vitamin which is essential for its antioxidative defense. According to recent scientific findings, the lung is not provided with vitamin E via LDL, as most of the other tissues are, but via HDL. This means that the supply of the lung with vitamin E decreases when LDL rises, HDL decreases and the supply of vitamin E remains constant. It is difficult to estimate to what extent this phenomenon is relevant for the resistance of the lung to oxidative stress. Some data show that vitamin E is accumulated in the lung during chronic stress. Thus, higher values of vitamin E are usually found in the alveolar macrophages of smokers. The concentration of vitamin E in the epithelial lining fluid (ELF) is, however, considerably lower.

Basically, the intravenous application of micronutrients can also be classified as nutritargeting. Specifically, when target tissues like the endothelium of the vessels must be supplied with a higher dose of vitamin E to ensure good protection during surgery with ischemia/reperfusion injury, the parenteral route is superior over the ''controlled'' oral route (Bartels *et al.*, 2004).

Vitamin E accumulates in the aortic endothelial cells after parenteral, but not oral, administration (Fig. 5.6).

The latter is due to the fact that vitamin E is integrated into VLDL by the liver and that the maximum concentration in the LDL is 7–10 molecules per LDL particle. Therefore, there is only a limited transfer of vitamin E from LDL to endothelial cells. An accumulation of vitamin E in endothelial cells can yet be seen as a preventive measure against the ischemia/reperfusion syndrome.

C. β-Carotene/carotenoids/fat-soluble antioxidants

Basically all fat-soluble compounds of the diet are absorbed and partially metabolized in the upper part of the small intestine. Afterwards, they are transported to their target organs via the systemic circulation. Therefore, it is impossible to deliver β-carotene to the lower parts of the intestine via the chymus. β-Carotene reaches this part of the intestines only after systemic absorption.

It was documented in several studies that high doses of β-carotene lead to a downregulation of ornithine-decarboxylase (ODC) in patients

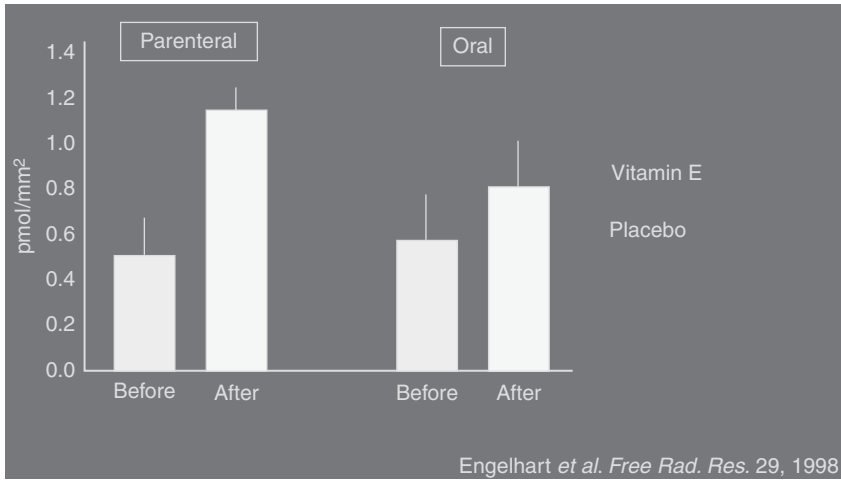


FIGURE 5.6 Accumulation of vitamin E in aortic endothelial cells following oral (3 × 1.0 mg) or parenteral (3 × 0.5 mg) administration during 3 days.

suffering from intestinal polyps. When supplementation (30 mg/day) was stopped, the ODC values in the tissue, that is, in the adenomatous polyps, rose again while β-carotene in the blood declined in parallel fashion (Phillips *et al.*, 1993). Since an increased activity of the ODC goes along with neoplastic changes, a reduction of ODC activity in these tissues is interpreted as tumor prevention. However, such an approach is problematic because β-carotene must be administered in very high doses. This may result in an unnecessary accumulation of this provitamin in other tissues and, eventually, lead to harmful effects.

Yet when β-carotene is coated with pectin (edible coating), the pectin prevents β-carotene — or other carotenoids as well as fat-soluble compounds — from being absorbed in the upper parts of the small intestine. As a consequence, these coated compounds can be transported via the chymus to the colon, where the pectin is broken down to short-chain fatty acids by bacteria. These fatty acids play an important role as growth regulators of the colonic mucosa cells. At the same time, the active agents, such as β-carotene, are released and can then be absorbed by the mucosa.

II. NUTRITARGETING AS A WAY OF BYPASSING ABSORPTION BARRIERS

Another interesting feature of nutritargeting is the ability to circumvent absorption or metabolization barriers during malabsorption, maldigestion, or when there is a lack of transport proteins, for example, tocopherol-

or retinol-binding protein. The identification of the different pathways that tissues and organs can use to accumulate micronutrients is one of the fundamental prerequisites to administer nutrients selectively. Presently, however, only very few mechanisms by which organs can be provided with micronutrients via nutritargeting in a controlled way have been identified.

The provision of fat-soluble vitamins and lipids is difficult, if not impossible, in various diseases. This is especially true for diseases that are accompanied by a lot of oxidative stress, for example, mucoviscidosis. The requirements of fat-soluble antioxidative substances are certainly high in these cases and can barely be covered by intramuscular injections because fat-soluble vitamins can hardly, if at all, be absorbed from oily preparations. Alternatively, the vitamins can administered via the buccal mucosa: the fat-soluble substances have to be packaged in such a way that they can be transported in a watery compartment and are thus able to largely dissolve in the saliva. When they have an adequate size, they can then penetrate the buccal mucosa. One approach is the development of the so-called nanocolloids, that is, particles with a polar nucleus, in which the fat-soluble vitamin is dissolved, and an apolar wrapping (monolayer). This structure makes an oral application of fat-soluble substances possible. First tests demonstrated that vitamin A palmitate, α-tocopherol, as well as coenzyme Q_{10} are thus able to enter the systemic circulation via the buccal mucosa.

A. Digestion and intestinal absorption of fat-soluble dietary components

Various factors are required for regular fat digestion. Sublingual lipase and eventually a gastric lipase — which are both stable in an acidic environment — start digesting dietary fats in the stomach. In the intestine, pancreatic bicarbonate as well as bile acids are essential for emulsification of fats and fat-soluble substances which are then cleaved by pancreatic lipases. The cleavage products are incorporated into micelles and can then penetrate the unstirred water layer (UWL) which covers the intestinal surface. There, they can deliver the cleavage products of dietary fats as well as fat-soluble substances (e.g., carotenoids, vitamin E, vitamin A) to the luminal surface of the enterocytes.

B. Fat malassimilation

The regular digestion of dietary fats and fat-soluble substances and/or their absorption via the lipid route is compromised in the frame of various diseases such as cystic fibrosis, short bowel syndrome, cholestasis, and chronic inflammatory bowel diseases.

C. Bioavailability of dietary, emulsified and solubilized fat soluble vitamins

Fig. 5.7 illustrates how the absorption of dietary fat-soluble substances via the lipid route is affected when lipids are insufficiently emulsified and incorporated into micelles. When fat-soluble vitamins are provided in stable water-soluble solubilizates (diameter of the micelles: 20–50 nm), they can directly permeate through the UWL and reach the membrane of the enterocytes where they can be absorbed. The block in the lipid route can thus be circumvented via the aqueous route. Fat particles in emulsions are as well quite small (diameter: 500–5000 nm). Digestion and absorption of fat-soluble vitamins from emulsions might thus be enhanced as well. The fat particles are, however, larger than those in the water-soluble form and therefore do not directly diffuse through the UWL. Furthermore, emulsions are more unstable toward changes in pH and might disaggregate in the acidic environment of the stomach, while the water-soluble micelles are more pH stable.

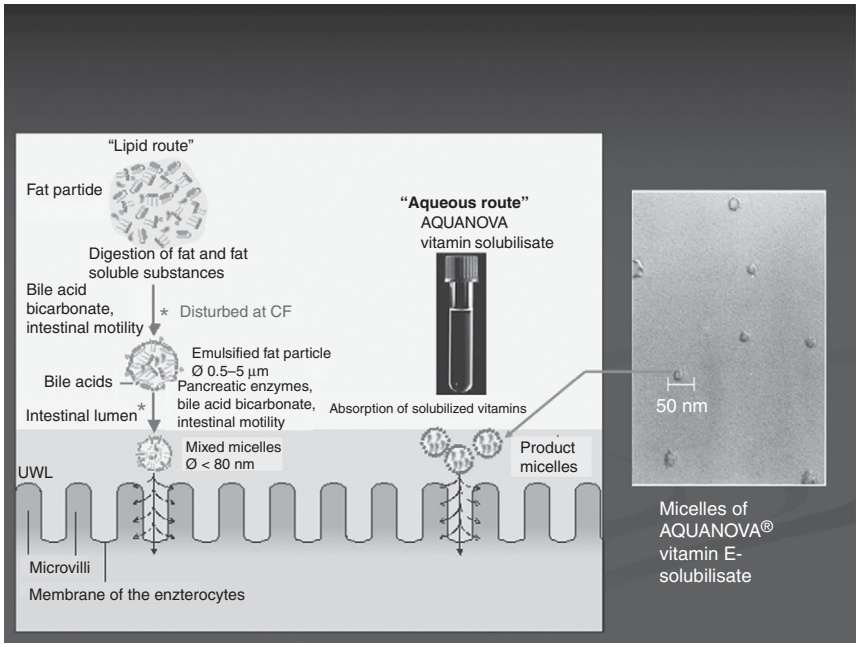


FIGURE 5.7 Absorption of dietary fat-soluble substances via the lipid route affected by insufficiently emulsified and micellised lipids.

D. Comparative bioavailability study (water-soluble micelles vs a regular supplement)

In a study with 14 healthy adult volunteers, we have assessed the bioavailability of d-α-tocopheryl acetate solubilizate (100 IU in form of a jelly baby; manufacturer of the solubilizate: Aquanova German Solubilisate Technologies GmbH, Darmstadt/Germany) in comparison to a regular supplement with the fat-soluble form of d-α-tocopheryl acetate (Back *et al.*, 2005). Four hundred-microgram crystalline vitamin C were administered together with vitamin E at all days either via the jelly babies or diluted in water. Vitamin C was added to the jelly babies in order to complement the ''antioxidant cocktail'' and furthermore, supplying a water-soluble vitamin served as a control measure. Since vitamin C is absorbed via another mechanism than the fat-soluble vitamins, no differences between the reference and study supplements should be observed between the 3-study days.

At all study days, subjects had venous blood samples taken after fasting for at least 12 h. Afterwards, the vitamins were administered. On day 0, the subjects were instructed to suck the jelly baby slowly and keep it in the mouth as long as possible in order to achieve a more or less constant salivary vitamin concentration. On days 10 and 20, the jelly baby or supplement, respectively, was swallowed with some water. Further venous blood samples were taken 1, 5, 15, 30, 60, 180, 240, 300, and 320 min after ingesting the vitamins.

Table 5.1 gives a survey of the area under the curve (AUC) of α-tocopherol and vitamin C at days 0, 10, and 20. There were no significant differences in the AUCs of vitamin C between day 0, 10, and 20, yet there was a significant difference between AUCs of α-tocopherol on day 0 and 20.

In order to assess the magnitude of the change in plasma concentrations after ingestion of the jelly babies and reference supplement, the difference between maximum and minimum plasma concentrations was calculated.

For vitamin C, plasma concentration was always about 40 μmol/liter and not significantly different between study days. There was, however, a significant difference between day 0 and day 10, respectively, versus day 20 in the plasma concentration for α-tocopherol.

In summary, our bioavailability study provided for the first time data for the short-term bioavailability of α-tocopherol solubilizate in comparison to regular fat-soluble preparations. Our results pointed to a higher short-term bioavailability of vitamin E in micelles versus fat-soluble forms of this vitamin in healthy adult volunteers both with regard to AUCs and with regard to maximum increases in plasma vitamin concentrations.

TABLE 5.1 Comparison of AUCs

	AUC day 0	AUC day 10	AUC day 20	*p*
α-tocopherol [(μmol/liter) × h)]	20.1 (10.9–58.1)	20.7 (9.8–39.5)	13.5 (6.9–30.6)	day 0 versus day 20: $p = 0.016$
β-carotene [((μmol/liter) × min)]	20.5 (4.2–86.7)	11.6 (2.7–32.0)	4.4 (0.38–25.0)	day 0 versus day 20: $p = 0.016$
Vitamin C [(μmol/liter) × h)]	145.0 (77.4–196.7)	131.2 (47.7–206.4)	118.0 (53.3–220.6)	NS

In a case study, Traber *et al.* (1986) reported that they successfully increased tocopherol in plasma and adipose tissue in a child with congenital hepatic cholestasis by oral administration of vitamin E as TPGS (a water-soluble form of vitamin E: tocopheryl succinate polyethylene glycol 1000) (100-mg tocopherol/kg body weight per day), while tocopheryl acetate emulsified with medium-chain triglycerides and polysorbate 80 did not have that effect. By administering the same form of vitamin E in a daily dose of 4000 IU, normal vitamin E plasma concentrations and increased adipose tissue α-tocopherol concentrations could be achieved in a 71-year-old patient with severe fat malabsorption and vitamin E deficiency (secondary to short-bowel syndrome) (Traber *et al.*, 1994).

Most of the above-mentioned bioavailability, intervention, and case studies came to the conclusion that water-miscible or water-soluble preparations of fat-soluble vitamins were superior to regular supplements. Based on the evidence from our own bioavailability study as well as from the studies mentioned above, it therefore seems justified to assume that fat-soluble vitamin deficit patients with fat maldigestion and/or malabsorption can be corrected more efficiently by using water-soluble as opposed to fat-soluble preparations. Another advantage of water-soluble preparations in general might be that lower daily doses are required when compared to fat-soluble preparations to achieve the same results.

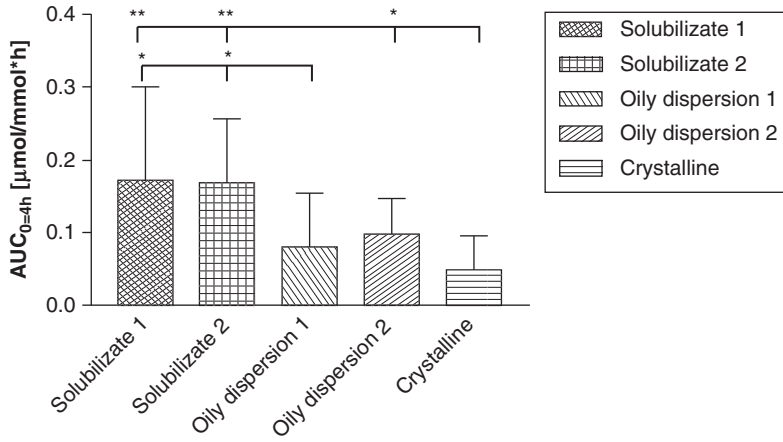
E. Coenzyme Q10 (CoQ_{10})

Since the bioavailability of CoQ_{10} in humans is relatively low due to its lipophilic nature and large molecular weight, several formulation technologies exist to improve its bioavailability from dietary

supplements (and therapeutics). Different Galenic preparations are available: crystalline CoQ_{10} powder in hard gelatine capsules, as well as oily dispersions and solubilizates of CoQ_{10} in soft gel capsules. These formulations largely differ in their bioavailability of CoQ_{10}. In our study, the main interest was to compare CoQ_{10} supplements (market standard formulations) with a novel solubilizate formulation of CoQ_{10} (Solu? Q10) based on polysorbates (Schulz *et al.*, 2006).

Since CoQ_{10} underlies biologic regulation and metabolism, we focused on early absorption parameters (0–4 h after ingestion). Additionally, long-term accumulation in blood and tissue was examined after consecutive dosing during 2 weeks.

As shown in Fig. 5.8, both solubilizate formulations showed equal $AUC_{0-4\ h}$ levels with 0.17 ± 0.13 µmol/mmol × h and 0.17 ± 0.09 µmol/mmol × h, respectively, and both were highly significantly superior to crystalline CoQ10 ($p < 0.01$). Both preparations also showed superiority over oily dispersion 1 (0.08 ± 0.07 µmol/mmol × h; $p < 0.05$), but missed statistical significance over oil dispersion 2 with 0.10 ± 0.05 µmol/mmol × h. Differences between formulations in $AUC_{0-12\ h}$, C_{max}, and T_{max} missed statistical significance. As expected, crystalline CoQ_{10} led to lowest C_{max} and highest T_{max} values. In general, solubilizates



Data are means ± SD
* p < 0.05 (solubilizates vs. oily dispersion 1; oily dispersion 2 vs. crystalline)
** p < 0.01 (solubilizates vs. crystalline)

FIGURE 5.8 Bioavailability of 60-mg CoQ_{10} single dosing (early absorption). $AUC_{0-4\ h}$ (µmol/mmol × h) after a single dose of 60-mg CoQ_{10}. Data are expressed as mean ± SD. Differences between formulations were tested with ANOVA and post hoc test Student-Newman–Keuls test. * $p < 0.05$ (both solubilizates vs oily dispersion 1 and oily dispersion 2 vs crystalline); ** $p < 0.01$ (both solubilizates vs crystalline).

reached higher levels and faster absorption rates compared to oily dispersions and crystalline CoQ_{10}.

After multiple dosing of CoQ_{10} for 14 consecutive days, a significant increase in plasma CoQ_{10} concentrations was seen in all groups ($p < 0.01$). The highest mean increase was reached by solubilizate 1. As depicted in Fig. 5.9, after 1 week of supplementation, solubilizate 1 seemed to have already reached a plateau level in plasma, whereas, for the other preparations, a further slight increase could be observed in the second week of supplementation. Looking at $AUC_{0–14\ day}$, the relative bioavailability of solubilizate 1 was 142% compared with crystalline CoQ_{10}, followed by oily dispersion 2 (131%), solubilizate 2 (107%), and oily dispersion 1 (89%).

Intracellular CoQ_{10} levels in BMC were analyzed independent of formulations. A significant increase ($p = 0.0282$) in intracellular CoQ_{10} content was observed in volunteers with baseline levels of <12 pmol/μg DNA (Fig. 5.10). Within this group, the correlation between plasma and intracellular CoQ_{10} status pre- and postsupplementation was evaluated and found to be significant with $r = 0.2659$ and $p = 0.0164$ (Pearson correlation coefficient). An increase of CoQ_{10} levels in plasma over time was more prominent compared to BMC. Volunteers starting with very

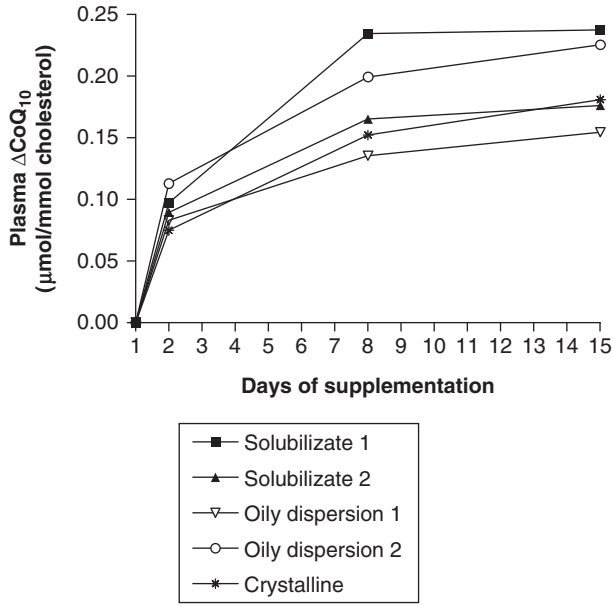


FIGURE 5.9 Increase in plasma CoQ_{10} concentrations (μmol/mmol cholesterol) after 1, 7, and 14 consecutive days of supplementation (60 mg/day). Means are plotted after baseline correction and correction for total cholesterol.

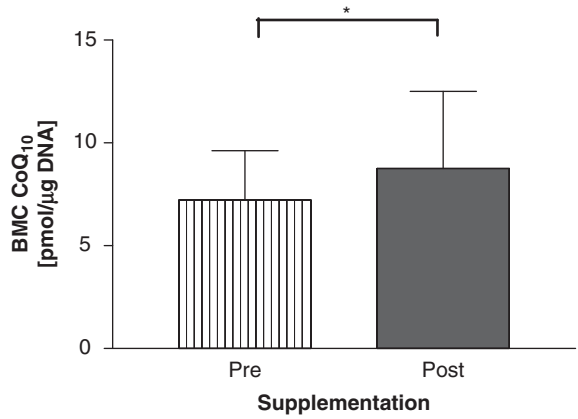


FIGURE 5.10 Intracellular CoQ_{10} content in BMC pre- and postsupplementation (14 days). Values are expressed as mean ± SD. Volunteers with baseline levels <12 pmol/µg DNA were pooled ($n = 41$); *$p < 0.05$ paired *t*-test.

high intracellular concentrations of CoQ_{10} leveled off normal values within 2 weeks of supplementation, indicating that there might be regulatory biologic systems within the physiologic range.

The superiority in early uptake is also reflected in calculating $AUC_{0–4\ h}$. Both solubilizates indicate clear superiority over oily dispersions and crystalline CoQ_{10} ($p < 0.01$) in this time frame. This might be explained by the structure of the water-soluble forms, small enough to be directly incorporated into the intestinal border. For preparation of a solubilizate, the detergent polysorbate 80 is used. Nerurkar *et al.* (1996) showed that permeability of CaCo-2 cells is enhanced by polysorbate 80 due to inhibition of an apically polarized efflux system. Additionally, Seeballuck *et al.* (2004) showed in an *in vitro* model with CaCo-2 cells that polysorbate 80 stimulates the secretion of triglyceride-rich lipoproteins (chylomicrons). Chylomicrons are the lipoprotein fraction mainly responsible for transport of lipophilic substances out of the intestines into the lymphatic system.

Future aspects: a step to selectively target vitamin E to cells (fibroblasts) was achieved by creating functionalized microemulsions. For α-tocopherol targeting to skin fibroblasts the RGD [motif of fibronectin that is chosen for the α-tocopherol targeting to skin fibroblasts and is the binding sequence (ligand) to integrin subunit α5 β1]-motif [GRGDSPA (synthetic peptide derived from the fibronectin receptor binding protein)] of fibronectin was chosen, which is the binding sequence (ligand) to integrin subunit α5β1. The significant higher uptake after 60 min of the peptide-functionalized microemulsion versus microemulsions without

the peptide implies the functionality of the ligand (Kramer, 2005). This kind of peptide functionalization might be a future and promising step to target nutrients selectively to cells and tissues.

III. CONCLUSION

With increasing knowledge about the role and the mode of action of micronutrients, it becomes increasingly important to transport these substances in a controlled way to the tissues to be protected. The technology developed for drug targeting can be adopted to the specific requirements in nutritargeting and organospecific accumulation can thus be rendered either possible or impossible. This option could play an important role, for example, in cancer therapy, when the healthy tissue has to be protected against the side effects of the therapy but at the same time the tumor has to be cut off from the supply. Radiotherapy, hyperthermia, and the photodynamic therapy as well as some chemotherapeutics work through the generation of free radicals. Thus, the accumulation of radical scavengers such as the vitamins C and E or carotenoids in the tumor is not desirable (Biesalski and Frank, 2002).

On the other hand, an accumulation of these radical scavengers in the healthy tissue could provide protection against therapy-induced damages.

There is a wide spectrum for the application of nutritargeting. At the moment, it is essential to classify organs and tissues which selectively accumulate specific micronutrients. This approach ensures that, in the future, risk groups can be supplied with the required substances much more effectively than presently.

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