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ORIGINAL CONTRIBUTION

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Solubility, uptake and biocompatibility of lutein and zeaxanthin delivered to cultured human retinal pigment epithelial cells in tween40 micelles

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Abstract Carotenoids lutein and zeaxanthin are proposed to protect ocular tissues from free-radical damage that can cause cataract and age-related macular degeneration (AMD). They accumulate selectively in the lens and macular region of the retina. Changes in the retinal pigment epithelium are characteristic in AMD. Efficient uptake is essential to study the intracellular effects of carotenoids in cell cultures. For in vitro experiments carotenoids are often dissolved in organic solvents like tetrahydrofuran (THF), dimethylsulfoxide (DMSO) and *n*-hexane, but difficulties have been associated with these application methods. Recently, O'Sullivan et al. (SM O'Sullivan et al., Br J Nutr 91 (2004) 757) developed a method whereby carotenoids could be delivered to cultured cells without the cytotoxic side effects often observed when organic solvents are used. We modified this method and investigated the effects of different carotenoid-formulations (ethanol/Tween40, methanol/tween40 and acetone/Tween40) on the uptake of lutein and zeaxanthin by differentiated ARPE-19 cells, cell viability and the expression of the "stress" gene HO-1, which is easily induced by a range of stimuli including chemical and physical agents. Micelle formulations prepared with ethanol/Tween40 resulted in the lowest LDH release, the highest carotenoid uptake and the lowest stress response (changes in HO-1 mRNA expression).

Key words lutein – zeaxanthin – age related macular degeneration (AMD) – retinal pigment epithelium (RPE) – micellation

Introduction

Age-related macular degeneration (AMD) is the leading cause of impaired visual function in people over 60 years in the western world. The outer layers of human retina containing photoreceptor outer segments and retinal pigment epithelium (RPE) might be primary targets for free radicals and reactive oxygen species mainly formed by photosensitised reactions [1]. There is currently much interest in the possibility that oxidative stress causes changes in RPE and subretinal layers associated with RPE, aging and retinal damage [2]. Oxidative stress in the retina can be caused by light exposure, which has been implicated in the pathogenesis of AMD and other retinal degenerations [3]. Photooxidation requires the presence of a photosensitiser. One of the significant photosensitisers in the retina is lipofuscin, which accumulates with age, and is highest in the elderly [4]. The antioxidant and light

absorptive properties of the dietary carotenoids, lutein and zeaxanthin have been implicated to their possible protective role against AMD. Carotenoids are fat-soluble phytochemicals present in fruits and vegetables. They are known for their high efficiency in quenching singlet oxygen and triplet excited states of photosensitizing dye molecules [5]. In vivo, lutein and zeaxanthin are incorporated into micelles after digestion and subsequently are packaged into chylomicrons for the transport to the liver, where they are transferred to VLDL and delivered to extra-hepatic tissues. Because it is difficult to deliver carotenoids to cells in culture, they are often dissolved in organic solvents like tetrahydrofuran (THF), dimethylsulfoxide (DMSO), ethanol and *n*-hexane. A number of difficulties have been associated with these solvents, including instability and insolubility, precipitation of the carotinoids in aqueous medium (cell culture medium), changes in gene expression patterns due to the organic solvent and cytotoxic side effects of the organic solvents.

Recently, O'Sullivan et al. [6] developed a system whereby carotenoids can be delivered to cells in culture without above-mentioned adverse effects. They have used two commercial emulsifiers, Tween40 (polyoxyethylene sorbitan monopalmitate) and Tween80 (polyoxyethylene sorbitan monooleate) to dissolve fat-soluble phytochemicals for delivering these substances to cultured cells. We modified this method and adapated to our cell system, human retinal pigment epithelial cells (ARPE-19). Specifically, ARPE-19 cells are capable of displaying a more differentiated phenotype than most other RPE in vitro models [7]. To preserve many of the phenotypic characteristics, which are exhibited by RPE in vivo, post-confluent ARPE-19 cells were allowed to differentiate for 6–8 weeks in cell culture. It has been demonstrated, that ARPE-19 cells grown for several weeks on plastic synthesise their own matrix that is similar to the basement membrane found in vivo [8]. These cells showed an almost similar mRNA expression profile when compared to native RPE [9, 10]. Using differentiated ARPE-19 cells, we analyzed the solubility, uptake and biocompatibility of lutein and zeaxanthin. If micelles containing lutein and zeaxanthin are feasible for investigating, we will study the role of these carotenids against photooxidative stress, which is believed to be one of the critical risk factors of AMD.

Materials and methods

Materials

All chemicals were obtained from Sigma (Deisenhofen, Germany) unless otherwise indicated. Cell culture materials were purchased from Biochrom (Berlin, Germany) or Greiner (Frickenhofen, Germany).

Cell culture and differentiation of ARPE-19 cells

ARPE-19 cells (ATCC CRL-2302) were plated at a density of 25,000 cells/cm^2 and maintained in culture for 3 days for undifferentiated cultures, or 6–8 weeks for differentiated cultures. ARPE-19, a non-transformed human diploid RPE cell line that displays many of differentiated properties typical to the RPE in vivo, exhibit a number of morphological and biochemical indications of differentiation when cultures are allowed to grow to post-confluent densities for several weeks [11]. At this stage, ARPE-19 cells express RLBP1 and RPE65, both of which are synthesized by differentiated RPE in vivo [12]. Both undifferentiated and differentiated ARPE-19 cells were grown in DMEM-HAM's F12-medium supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS) at 37°C in a humidified 5% CO_2 atmosphere. Cells were withdrawn from serum for 24 h prior to using them for indicated treatments.

Preparation of synthetic micelles containing lutein und zeaxanthin (0.1% Tween40 and 0.01% Tween40)

All-E-zeaxanthin and all-E-lutein were gifts from DSM Nutritional Products Ltd, R&D Analytical Research Center (Basel, Switzerland). They were dissolved in THF at a concentration of 10 mg/ml and concentrations of stock solutions (diluted in ethanol 1:2000–1:4000) were determined spectrophotometrically (UV2, Unicam, Kassel, Germany) using published extinction coefficients for lutein 145100 l/(mol × cm) (λ = 445 nm), and zeaxanthin 144500 l/(mol × cm) (λ = 450 nm). Distinct aliquots of lutein and zeaxanthin solutions were dried under a stream of N_2 gas and then micellated either in ethanol/Tween40, acetone/Tween40 or methanol/Tween40 (4:1 v/v). These solutions were concentrated under a stream of N_2 gas and dissolved in cell culture medium. The end concentration of the Tween40 in the culture medium was 0.1%.

In a second set of experiments, the concentration of Tween40 was reduced to 0.01%. Lutein and zeaxanthin were micellated in ethanol/Tween40 (31:1 v/v). These solutions were concentrated under N_2, dissolved in 1 ml of medium and diluted 1:10 in cell culture medium prior to applying to ARPE-19 cells. The final concentration of the Tween40 in cell culture medium was 0.01%.

Uptake of lutein and zeaxanthin by differentiated ARPE-19 cells

Differentiated ARPE-19 cells were incubated for 24 h in culture medium containing lutein or zeaxanthin in the form of micelles (ethanol/Tween40) at concentrations ranging from 0 to 10 μM. The final concentration of the vehicle (Tween40) was either 0.1% or 0.01% for all the carotenoid concentrations. Micelles without carotenoids served as vehicle controls.

Extraction and analysis of lutein and zeaxanthin from differentiated ARPE-19 cells

Intracellular concentrations of lutein and zeaxanthin in ARPE-19 cells were analysed by reversed-phase HPLC [HPLC serial HP 1100 (Hewlett Packard, Waldbronn, Germany)] equipped with a diodearray detector. After incubating differentiated ARPE-19 cells with the synthetic micelles containing lutein and zeaxanthin, cells were washed twice with warm PBS^- (without calcium and magnesium), detached from cell culture dishes by 0.05% trypsin (w/v) and sedimented in glass tubes (1600 × *g* for 5 min). Thereafter, lutein and zeaxanthin were extracted by methanol/dichloromethane (1:3 v/v). The extraction was repeated three times and pooled fractions were dried in a rotary evaporator under vacuum and subsequently redissolved in 500 μl acetonitrile containing 0.5 mM butylated hydroxytoluene.

Lutein and zeaxanthin were quantified by HPLC according to Schlatterer et al. [13]. The injection volume was 40 μl. The samples were eluted using a mobile phase composed of eluant A (methanol) and eluant B (TBME (*t*-butyl methyl ether)/methanol/water [90:6:4 (v/v/v)] with gradient by 0 min (1% B), 10 min (10% B), 20 min (30% B), 30 min (100% B), 35 min (1% B), 40 min (1% B) at a flow rate of 1 ml/min. All chemicals for HPLC analysis were purchased from Acros organics (Schwerte, Germany). Carotenoids were detected at 450 ± 4 nm (reference wavelength: 550 ± 20 nm). The column (YMC C30-reverse-phase, 250 × 4.6 mm, 5 μm) and the precolumn (YMC C30-reverse-phase, 10 × 4 mm, 5 μm) were purchased from YMC Europe (Schermbeck, Germany). Results were obtained and analysed by HP ChemStation software (Rev.A.04.02).

Induction of photodynamic damage in differentiated ARPE-19 cells by Merocyanine 540 (MC540)

Differentiated ARPE-19 cells were incubated for 4 h with 55 μM MC540 at 37°C in the dark (Invitrogen, Karlsruhe, Germany) with medium containing 2.5% FCS. Then, medium was replaced by phenol red-free DMEM-HAM's F12 medium (1:1) (Promocell, Heidelberg, Germany) without FCS. Thereafter, cells were illuminated with a light intensity of 70 mW/cm^2 and a light dose of 4 J/cm^2. The radiation source was a halogen lamp emitting wavelengths over the range 420–1400 nm (hydrosun® PIRA-radiator, Hydrosun Medizintechnik GmbH, Muellheim, Germany). By using a band-pass filter, cells were illuminated with a wavelength range encompassing the activation wavelengths for MC540 (340–800 nm). Light dosimetry was performed using a calibrated radiometer/photometer (IL1400A, International Light, Newburyport, MA). At this illumination condition, a cell survival rate of about 35% was achieved for assessing a putative protective effect of micelles (Tween40) on phototoxicity. Cell viability was measured by LDH and MTT assays after 24 h illumination.

Measurement of cell viability

LDH assay

Lactate dehydrogenase (LDH) is an oxidoreductase, which catalyses the interconversion of lactate and pyruvate. LDH is measured to evaluate the presence of tissue or cell damage. The release of LDH from cells into the medium was measured spectrophotometrically using the LDH cytotoxicity detection kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's protocol. ARPE-19 cells treated with 1% Triton-X-100 for 24 h served as a positive control of the test system.

MTT assay

The amount of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that is oxidised into purple formazan was measured spectrophotometrically. This oxidation takes place only when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells. For measuring cell viability, ARPE-19 cells were maintained for 2 h in medium containing 0.02% MTT reagent. Thereafter, cells were lysed in 1 ml solubilisation buffer (99.4 ml DMSO, 0.6 ml acetic acid (100%) and 10 g sodium dodecyl sulfate) and absorbance was read with a 590 nm filter (reference filter: 660 nm) using a BioKinetics Microplate reader (Reader EL 340, Bio Tek® Instruments, Vermont, USA). ARPE-19 cells treated with 1% Triton-

X-100 for 24 h served as a positive control of the test system.

Detection of RLBP1, RPE65, and HO-1 mRNA expression by real-time PCR

The mRNA expression of RLBP1, RPE65, and HO-1 was measured quantitatively by real-time PCR (iCycler, Bio-Rad, Munich, Germany). Total RNA isolation was done with RNeasy mini kit (Qiagen, Hilden, Germany). 1 μg of total RNA was transcribed into cDNA using i Script cDNA Synthese Kit (Bio-Rad, Munich, Germnay) and oligo dT-primers (end volume: 20 μl) according to manufacturer's instructions. The following primers were used for the amplification: RLBP1; 5′-CTG CTG GAG AAT GAG GAA ACT-3′ and 5′-CAC ATT GTA GGT CGT GGT GAA-3′; RPE65: 5′-GTT TTG GTC TGA CTC CCA ACT-3′ and 5′-GGT GTT GAT GTG ATG GAA GAG-3′; HO-1: 5′-GGT GAT AGA AGA GGC CAA GAC-3′ and 5′-GCA GAA TCT TGC ACT TTG TTG-3′. 18s rRNA: 5′-ACA CGG ACA GGA TTG ACA GA-3′ and 5′-GGA CAT CTA AGG GCA TCA CA-3′. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) according to manufacturer's instructions. Briefly, 1 μl of cDNA was added to the master mix (12.5 μl 2 × iQ SYBR Green Supermix, 0.25 μl of each primer [25 pmol/μl], 11 μl RNAse-free water). A calibration curve was set up in parallel in duplicates with each analysis using PCR fragments of the target cDNA at concentrations ranging from 10^1 to 10^7 copies per sample. Negative water blanks were included in every analysis. A 3 min denaturation step at 95°C activated the Taq Polymerase followed by 30 or 40 PCR cycles in accordance with the following protocol: denaturation at 95°C (30 s), annealing at 57°C up to 61°C; (30 s), and elongation at 72°C (30 s). At the end of the PCR, a melt-curve analysis was performed by gradually increasing the temperature to 95°C to detect possible formation of primer–dimers. Data acquisition was performed during elongation step. After PCR run, the SYBR Green fluorescent signal was transformed into a relative number of copies of target molecules. Differences in cDNA amounts were normalized to the expression of the 18s rRNA.

Statistical analysis

Results are expressed as means ± SEM unless stated otherwise. Differences between groups were assessed by the two-tailed Mann–Whitney test for unpaired samples. The significance level was set at $\alpha = 5\%$ for all comparisons.

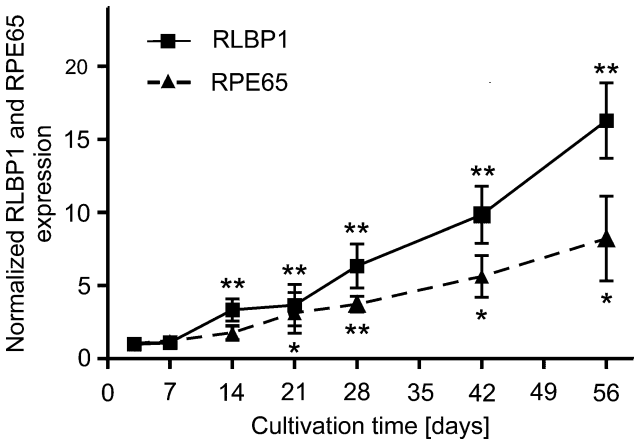

Fig. 1 Expression of RPE-differentiation specific genes (RLBP1 and RPE65) as a function of time in culture. Total RNA was isolated at various time periods and transcribed into cDNA, which was used as a template for quantitative real-time PCR (qPCR). Differences in cDNA amount were equalised by expression of 18s rRNA. The expression of RLBP1 and RPE65 is presented in relation to undifferentiated ARPE-19 cells (day 3), which were equalised 100%. Results represent mean ± SEM of four independent experiments; * $P < 0.05$; ** $P < 0.01$ v.s. undifferentiated ARPE-19 cells

Results

Expression of RLBP1 and RPE65 mRNA as a function of ARPE-19 differentiation

ARPE-19 cells were cultured for a few weeks for evaluating the expression of RLBP1 and RPE65. Both genes are specific markers, which are highly expressed in differentiated RPE cells [11, 14]. RLBP1 mRNA was significantly upregulated ($P < 0.01$) at day 14 and increased continuously with the age of the culture (Fig. 1). RPE65 mRNA was also upregulated as a function of RPE cell differentiation starting at day 21 ($P < 0.05$) (Fig. 1). We chose a culture-time of 6–8 weeks as a standard period for differentiating ARPE-19 cells, which were considered as differentiated and used for all our subsequent experiments.

Effects of micelle preparation on differentiated ARPE-19 cells

Regularly, after administration of lutein and zeaxanthin dissolved in THF into the aqueous cell growth medium, formation of carotenoid precipitates were observed which looked like a thin oily film on the aqueous cell culture medium and after exhausting the medium it could be found on the surface of cells (Fig. 2A, B). This oily film could not be removed, even after diverse washing steps so that subsequent determination of intracellular lutein or zeaxanthin would be misleading. Therefore, we chose

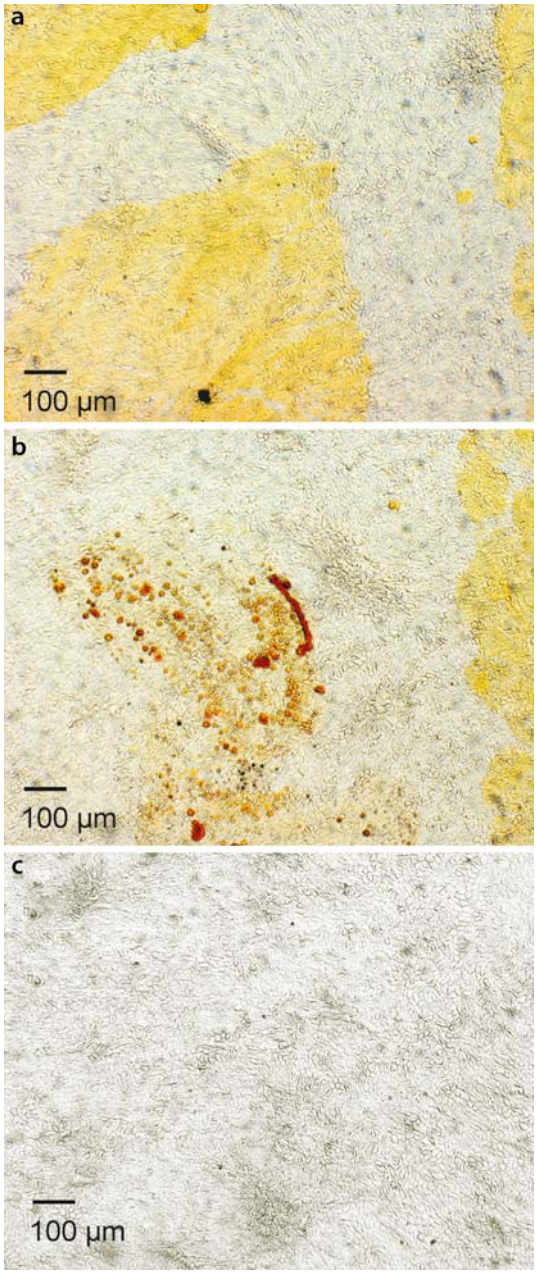

Fig. 2 Light microscopic pictures of carotenoid-treated ARPE-19 cells. ARPE-19 cells were incubated with 5 μM lutein (**A**) or 5 μM zeaxanthin (**B**) delivered in THF. Carotenoid precipitation was observed as soon as lutein or zeaxanthin dissolved in THF was administered into the aqueous cell growth medium, which looked like a thin oily film, lying on top of the aqueous cell culture medium. After exhausting the medium, particles of this oily film were found on the surface of the cells. The oily film adheres to the cells and could not be removed by multiple washing steps. This effect was not observed when lutein and zeaxanthin were delivered in ethanol/Tween40 micelles (**C**)

an alternate method for delivering lutein and zeaxanthin to cultured cells, which was recently described by O'Sullivan et al. [6]. We prepared three different formulations: acetone/Tween40, methanol/Tween40, or ethanol/Tween40 to verify which of these synthetic micelle preparations may be the most suitable for cells under our experimental conditions. ARPE-19 cells were incubated in the delivery vehicles at a concentration of 1 ml/l (0.1%) for 24 h. A clear increase of LDH in the culture medium was found with all three vehicle preparations greatest with methanol/Tween40 (Fig. 3A). The lowest LDH release was observed for micelles that were prepared with ethanol/Tween40. Because ethanol/Tween40 vehicles delivered substantially more carotenoids to the cells compared with acetone/Tween40 (Fig. 4A), and because of the higher cytotoxic effect of methanol/Tween40 vehicles, we used the ethanol/Tween40 formulation for all further experiments. No precipitation of carotenoids was observed with this method (Fig. 2C).

Cytotoxic effects of Tween40 concentration on differentiated ARPE-19 cells

To reduce the cytotoxic effects resulting from ethanol/Tween40 vehicles delivered in a concentration of 0.1% to the cells, we reduced the Tween40 concentration to 0.01%. As demonstrated in Fig. 3B, no cytotoxic effect was observed at this Tween40 concentration as assessed by the MTT assay. As observed in the LDH assay (Fig 3A), ethanol/Tween40 at a concentration of 0.1% showed a significant reduction in mitochondrial activity. To further assess the influence of Tween40 concentration on cytotoxicity, we analysed the mRNA expression of heme-oxygenase-1 (HO-1), a gene which is easily induced by a range of stimuli including chemical and physical agents [15]. ARPE-19 cells incubated with 0.1% Tween40 showed a significant induction of HO-1 mRNA expression, indicating a "stress" situation for the cells. In contrast, Tween40 at a concentration of 0.01% showed no significant induction of HO-1 mRNA expression (Fig. 3C). These results are consistent with the results found in the cell viability assay (Fig. 3B).

Effect of ethanol/Tween40 micelles on phototoxicity

In contrast to untreated cells (control), ethanol/Tween40 micelles at a concentration of 0.1% protected differentiated ARPE-19 cells against merocyanin 540-induced photocytotoxic effects (Fig. 3D). Lowering the concentration of ethanol/Tween40 micelles to 0.01% Tween40 abrogated this effect so that the cell damage was comparable to cells, which were irradiated without vehicle (Fig. 3D).

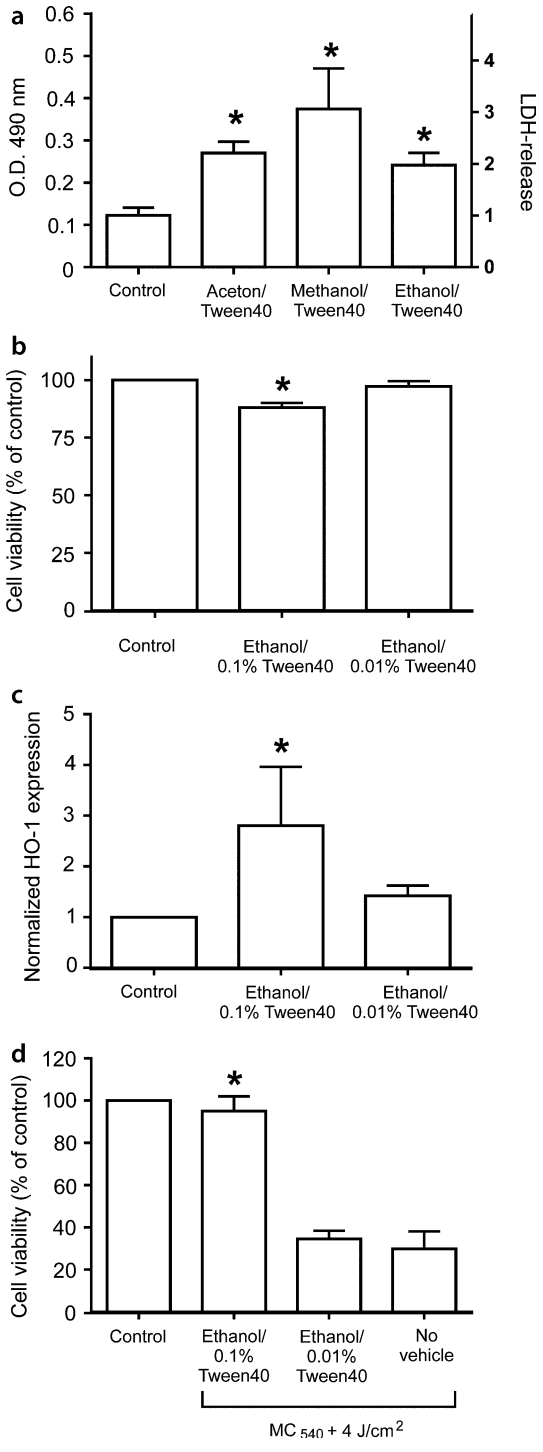

Fig. 3 Effects of micelle preparation on ARPE-19 cells. (**A**) Differentiated monolayers of ARPE-19 cells were incubated in the medium supplemented with different micelle preparations. The end concentration of Tween40 in medium was for all micelle preparations 0.1%. The effect of micelle preparation on cell viability was measured by the lactate dehydrogenase (LDH) assay. The unit of LDH-release is fold increase from control, whereby the control was equalized 100%. Values of four independent experiments are expressed as mean ± SEM. (**B**) Effect of micelle concentration (0.1% vs. 0.01%) on cell viability measured by the MTT assay. Values of four independent experiments are expressed as means ± SEM * $P < 0.05$ treated vs. control. (**C**) Effect of micelle concentration on mRNA expression of the stress related HO-1 gene. Gene expression was quantified by real time PCR. Mean ± SEM of four independent experiments is shown. * $P < 0.05$ treated vs. control. (**D**) Effect of micelle concentration on the efficiency of MC540 mediated photocytotoxicity. ARPE-19 cells were preincubated with MC540 and illuminated with a light intensity of 70 mW/cm^2 and a light dose of 4 J/cm^2. Cell viability was assessed by MTT assay. Mean ± SEM of four independent experiments is shown. * $P < 0.05$ vs. "no vehicle"

Cellular uptake of lutein and zeaxanthin

The cellular accumulation of lutein and zeaxanthin dissolved in ethanol/Tween40 (0.01%) was measured after 24 h post-treatment. Cellular concentrations of both carotenoids increased dose-dependently (Fig. 4B). Interestingly, differentiated ARPE-19 cells showed more affinity to zeaxanthin than lutein. The cellular zeaxanthin was significantly higher than that of lutein (Fig. 4B).

Discussion

The utilization of long-term cultures of ARPE-19 cells may be a useful in vitro model to study the retinoid and carotenoid metabolism and the regulation of gene expression in retinal pigment epithelial cells [11]. Therefore, we used differentiated ARPE-19 cells to examine the cellular uptake of lutein and zeaxanthin dissolved in Tween40. Polyoxyethylene sorbitan esters (Tweens) represent one type of non-toxic surfactants that have been used for the solubilisation of lipophilic drugs for oral, topical and ocular administration [16–20]. Recently, O'Sullivan and colleagues [6] developed a system for delivering lipophilic phytochemicals to cells in culture by the use of Tween40. We modified this method to supply long-term cultures of ARPE-19 cells with lutein and zeaxanthin. Furthermore, we analysed if this kind of in vitro supplementation of carotenoids may have unwanted secondary effects on the cell integrity in differentiated ARPE-19 cells. In addition, we wanted to test if this method is feasible for investigating the role of lutein and zeaxanthin in photooxidative stress or if there is an interaction between Tween40, membrane integrity and light.

First, we examined the effect of different organic solvents that are used for micelle preparation. The uptake of lutein or zeaxanthin from the synthetic micelle into the cells was most suitable if micelles were prepared with carotenoids that are dissolved in methanol (methanol/Tween40 formulation). A similar uptake of lutein and zeaxanthin from the micelle into the cells was observed by using the ethanol/Tween40 formulation. A 50% reduced uptake of lutein and

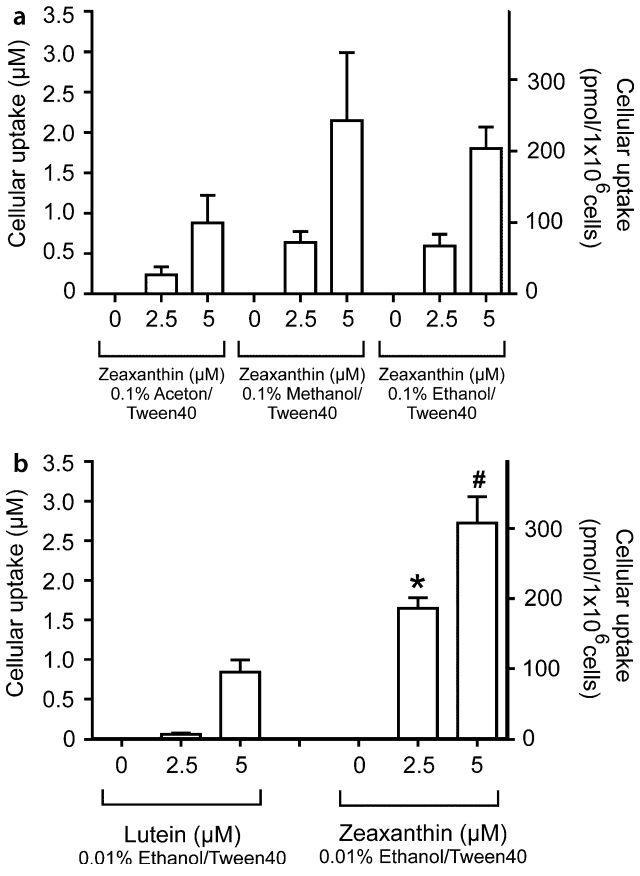

Fig. 4 (**A**) Effect of vehicle formulation on the uptake of zeaxanthin into differentiated ARPE-19 cells. For all vehicle formulations, the final Tween40 concentration was 0.1%. (**B**) Cellular uptake of lutein and zeaxanthin in differentiated ARPE-19 cells. Differentiated ARPE-19 cells were incubated in medium supplemented with Lutein and zeaxanthin in ethanol/Tween40 for 24 h at a final Tween40 concentration of 0.01%. Intracellular concentrations of both carotenoids were determined by HPLC analysis after extraction of lutein and zeaxanthin from differentiated ARPE-19 cells. Mean ± SEM of four independent experiments is shown. * $P < 0.001$ vs. 2.5 μM lutein and # $P < 0.01$ vs. 5 μM lutein

zeaxanthin from the micelle into the cells was observed by using acetone/Tween40 (Fig. 4A), the formulation which was originally applied by O'Sullivan [6]. In contrast to the study by O'Sullivan, we detected in our cell system a clear toxicity of the delivery vehicles to lipid membranes. All three micelle formulations showed a significant increase in the release of LDH from the cells into the medium indicating the cell membrane damage (Fig. 3A). Because of the low LDH release and the good uptake rate of zeaxanthin from the vehicle into the cells, we used the ethanol/Tween40 formulation for all further experiments. In a second step, we tried to reduce the side effects of the delivery vehicle by reducing the concentration of Tween40 from 0.1% to 0.01% without changing the amount of lutein and zeaxanthin offered for micellation. As seen in Fig. 4, the cellular uptake of zeaxanthin from the vehicle into the cells was not diminished by the reduction of the vehicle concentration, suggesting a higher loading efficiency of micelles with zeaxanthin under these conditions. Furthermore, the side effects and "stress" reaction (HO-1 expression) observed at a vehicle concentration of 0.1% were no longer detectable.

Further interest was to investigate the putative influence of the delivery vehicle on phototoxicity in the presence of a photosensitiser. As a model system we used the photosensitiser, merocyanin 540, to induce light damage [21]. The survival of differentiated ARPE-19 cells, subjected to merocyanin 540-mediated photodynamic action, was determined by the MTT assay. Surprisingly, we found a protective effect of the empty delivery vehicle (ethanol/Tween40) on phototoxicity when applied at a concentration of 0.1% although this concentration had the greatest membrane damaging effects. This may be a secondary effect, which is caused by an elevated release of merocyanin through the damaged membrane so that the intracellular concentration of the photosensitiser decreased upon time. This effect was abrogated when the vehicle concentration was reduced to 0.01%. A light absorbing effect of Tween40 (0.1%) could not be observed (data not shown).

In conclusion, ethanol/Tween40 vehicles were effective delivery vehicles for lutein and zeaxanthin to supply differentiated ARPE-19 cells with these carotenoids when the vehicle concentration does not exceed 0.01%. We used a concentration range from 2.5 to 5 μM for lutein and zeaxanthin, which had been approximately equal to the physiological levels in human serum. Bone et al. showed that 30 mg/d lutein or zeaxanthin supplementation increase the serum carotenoid concentration from 0.17 to 2.4 μM for lutein and from 0.1 to 0.56 μM for zeaxanthin [22]. Zeaxanthin was less absorbed than an equal lutein dose because zeaxanthin could be metabolized to lutein or/and uptake of zeaxanthin into the serum was lower than that for lutein [22, 23]. Furthermore, we compared the cellular uptake of lutein and zeaxanthin in the ARPE-19 cells with the levels of lutein and zeaxanthin in individual human ocular tissues. The level of lutein in the inferior RPE/choroid was 0.53 ng/20 mm^2 tissue area and in the macular retina 13.9 ng/20 mm^2 tissue area. The level of zeaxanthin in the inferior RPE/choroid was 0.32 ng/20 mm^2 tissue area and in the macular retina 19.1 ng/20 mm^2 tissue area (Bernstein et al.). In our in vitro study, we determined the following values after carotenoid supplementation of ARPE-19 cells: 1.27 ng/20 mm^2 cell area for 2.5 μM lutein, 20.1 ng/20 mm^2 cell area for 5 μM lutein, 39.5 ng/20 mm^2 cell area for 2.5 μM zeaxanthin and 65.2 ng/20 mm^2 cell area for 5 μM

zeaxanthin. The cellular levels of lutein and zeaxanthin in our in vitro study were approximated in the range of those in the human ocular tissues according to Bernstein et al.

In next set of experiments, we will study the effects of this physiological concentrations of lutein and zeaxanthin on photoxidative stress, which will be induced by merocyanin 540 and light irradiation. Because, light induced oxidative stress is believed to be a critical risk factor of AMD.

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