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Cell Culture Model for Examining Fat-Soluble Nutrient Absorption *In Vitro*

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Abstract Permeable support systems (PS) are employed in *in vitro* nutrient absorption studies but data are absent on their efficacy compared to conventional cell culture models (CONV). The *in vivo* absorption of fat soluble nutrients is influenced by its delivery vehicle, yet a fundamental understanding of the influence of the vehicle on cells in culture is lacking. We compared the efficacy of lutein absorption in Caco-2 cells cultured with CONV and PS, and examined the role of micelles, the physiological vehicle within the small intestine. After plating for 2 and 21 d to attain confluence and differentiation in CONV and PS, respectively, cells were treated with lutein in micelles or ethanol. After incubation, lutein in cell lysate, as well as apical and basolateral mediums, were quantified by HPLC-UV. After 24 h, cellular lutein in CONV was ≥460 and 8% greater in ethanol and micelle, respectively, than in PS. However, the intracellular AUC over time was only different for ethanol ($P \leq 0.05$). In PS, 0.15% of micellized lutein was secreted into the basolateral medium in contrast to 0.016% of lutein in ethanol. The absorption of lutein (uptake + secretion), independent of the vehicle, in CONV increased in a linear manner with dose (0.35 to 4 or to 14.6 µg/mL for ethanol or micelle, respectively), while that in PS peaked at 1.18 µg/mL. Caco-2 cells cultured in PS grow to display the phenotype and function of small intestine enterocytes and suggest this *in vitro* platform generates information closest to the natural physiology of the absorptive process. However, although the CONV has the physiology of colonic tissue, it appears to display a greater efficacy for lutein uptake by Caco-2 cells and so can provide a more rapid, preliminary method for nutrient absorption studies.

Keywords: *Caco-2 cells, lutein (PubChem CID: 5281243), cell culture model, micelle, ethanol (PubChem CID: 702)*

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1. Introduction

In vitro cell culture models present a useful means to study mechanisms of intestinal nutrient transport. The human intestinal Caco-2 cell line has been used extensively for studies examining nutrient and drug absorption [1,2,3]. Caco-2 cells originate from a human colonic carcinoma, and after differentiation, exhibit morphological and functional characteristics comparable to those of differentiated epithelial cells lining the mucosa of the small intestine. Differentiation to an enterocyte-like phenotype spontaneously occurs when the cells grow to confluency in conventional monolayer culture conditions [4,5,6]. However, during the early phases of differentiation, these cells express both colonocyte and enterocyte-specific proteins, such as surfactant protein A and α-1 antitrypsin, respectively [7]. As differentiation progresses, morphological and biochemical characteristics of enterocytes develop, including tight junctions, microvilli, enzymes and transport systems, along with the reduction of colonocyte-specific gene expression.

Caco-2 cells can be cultured on permeable cell culture support systems (PS) or conventional tissue cultures on treated plastic plates (CONV) for model absorption experiments. While both culture systems are commonly employed, there are important differences between them. Cells cultured in CONV remain in a proliferative stage due to constant cell detachment resulting from the intracellular fluid being transported to the basolateral space of cells [8,9]. PS allows investigation of cell permeability and transepithelial transport because of the complete establishment of functional tight junction assemblies between cells [5]. Further, the apical and basolateral surfaces of cells in the PS face the upper and lower compartments, corresponding to the intestinal lumen side and serosal side, respectively. However, data are absent on whether PS generates more efficient intestinal absorption and more physiologically relevant information than CONV.

The absorption of fat-soluble vitamins and carotenoids *in vivo* is influenced by the vehicle that carries the nutrient to the apical membrane of the enterocytes. During digestion, fat soluble nutrients are released from their food matrix and transferred to mixed micelles or, to a lesser extent, to vesicles such as liposomes [10,11] or associated with proteins such as β-lactoglobulin [12]. Once solubilized, uptake into the intestinal mucosa involves passive diffusion or facilitated transport through cholesterol transporters [13,14,15]. The final step in the absorption assimilates the

nutrient into chylomicrons and then secretes them into the lymph for systemic distribution. Aside from the major physiological vehicle, i.e., mixed micelles, fat soluble nutrients may also be delivered after they are incorporated into liposomes or water-miscible beadlets [16,17,18,19]. The organic solvents tetrahydrofuran (THF), dimethylsulfoxide (DMSO), and ethanol can be employed as delivery vehicles [20,21,22,23], but can present with serious limitations, such as instability, insolubility, and cytotoxicity.

The efficacy of nutrient intestinal absorption using Caco-2 cell monolayers grown in CONV and PS has not been systematically compared. We compared the absorption efficacy of lutein in these 2 systems because of the putative health benefits of lutein and research efforts to optimize its bioavailability [24,25,26]. We also tested 2 delivery vehicles that were employed to solubilize lutein, i.e., a synthetically prepared micelle that models the physiological vehicle within the small intestine and the organic solvent ethanol, which despite its intermediate solubility, is safe and commonly consumed in humans [27]. A better understanding of the properties of CONV vs. PS and the type of delivery vehicle therein can provide practical guidelines regarding *in vitro* approaches to the study of fat-soluble nutrient absorption.

2. Materials and Methods

2.1. Chemicals and Materials

β-Cryptoxanthin (BC, 97%), 1-oleoyl-*rac*-glycerol [monoolein (MO), 99%], 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (PC, 99%), 1-palmitoyl-*sn*-glycero-3-phospho-choline [lysolecithin, (LC), 99%], sodium glycodeoxycholate (GDC, 97%), sodium taurodeoxycholate hydrate (TDC, 97%), taurocholic acid sodium salt hydrate (TC, 95%), sodium oleate (OA, 99%) and bovine serum albumin (97%) were purchased from Sigma-Aldrich (St. Louis, MO). Advanced Dulbecco's Modified Eagle Medium (DMEM), 200 mM L-glutamine, penicillin-streptomycin (10,000 U/mL) were purchased from Gibco, Life Technologies Inc. (Grand Island, NY). Hyclone phosphate buffer saline (PBS) without Ca^{2+} or Mg^{2+}, Pierce RIPA buffer, and Pierce bicinchoninic acid (BCA) protein assay were purchased from Thermo Fischer Scientific (Rockford, IL). Multi-well tissue culture treated plates, Transwell® permeable supports, cell culture treated flasks, and sterile filters (0.22 µm pore size) were purchased from Corning Life Sciences (Tewksbury, MA). Caco-2 cells, fetal bovine serum, and trypsin-EDTA (0.25%) were purchased from ATCC (Manassas, VA). All other solvents were HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO). Purified lutein (70-75%) was a gift from Kemin Industries Inc. (Des Moines, IO).

2.2. Preparation of Lutein

Unmodified pure lutein in ethanol was added directly to serum-free medium, vortexed, and sonicated at room temperature for 2 min. Micelles containing lutein were prepared according to Chitchumroonchokchai et al. (2004) [28]. Briefly, MO, PC, and LC in chloroform (500, 200, and 200 µM, respectively), OA (1500 µM) in methanol, and lutein in ethanol were added to a conical glass tube and dried under N_2 gas at room temperature. Filtered sterilized serum-free medium containing 800, 450, and 750 µM GDC, TDC, and TC, respectively, were added to reconstitute the dry residue, and the resulting mixture was sonicated for 30 min at room temperature under red light to minimize photo-oxidation.

In the time course experiment, lutein concentration at 4 and 14.6 µg/mL for ethanol and micelles, respectively, was employed to evaluate the effect of the test vehicle and culture system on its absorption. The concentration of 14.6 µg/mL of lutein (~5 mg/d) was selected for micelles based on the consideration that it would be an adequate dose to quantify the magnitude of the absorption. Further, this concentration was calculated based on the assumption of a mean dietary intake of 1 mg/d lutein [29] and concurrent delivery of 2-3 L of water to the small intestine. Lutein at 4 µg/mL (~1.3 mg/d) was selected for testing with ethanol to assure lutein solubility and cell viability. Although the solubility of lutein in ethanol has been shown to be 300 µg/mL [27], we were unable to successfully prepare lutein in ethanol at >4 µg/mL. Further, the viability of Caco-2 cells in ethanol decreases when exposed to ethanol concentrations >7% [30]. In the dose-response experiment, the lutein concentration in the medium for the ethanol arm was 0.35, 1.18, and 4 µg/mL, equivalent to a low to average daily lutein intake (~0.11, 0.39, and 1.3 mg in 3 L intestinal water). The lutein concentration in the medium for the micelle arm was 0.35, 1.18, 4, and 14.6 µg/mL, equivalent to a low to high average daily lutein intake of ~0.11, 0.39, 1.3, and 5 mg in 3 L intestinal water.

2.3. Cell Culture

Caco-2 cells were maintained in advanced DMEM supplemented with 10% fetal bovine serum and 1% L-glutamine and the absence of antibiotics in a humidified incubator (Thermo Scientific Series 7000, Cambridge, MA) at 37°C and 5% CO_2. Cells between the 12^{th} and 27^{th} passages were used for all experiments.

To examine the uptake of lutein in CONV, cells grown in the medium in the absence of antibiotics were seeded at a density of 7 x 10^4 cells/well on 24-well tissue culture treated plates (1.9 cm^2 growth area/well). Cells were grown for 48 h to attain ~80% confluence. At the beginning of each experiment, serum-containing media was removed and replaced with 1 mL of lutein enriched serum-free media. Since cells do not adhere tightly to the plates, washing of the cells with PBS to remove any residual serum was avoided. Serum-free media was utilized to remove any variability in performance [4]. The range of the time course (0-48 h) was selected based on the data from pilot experiments of cell uptake, which showed that the maximal uptake was not reached by 24 h but plateaued by 48 h (results not shown). Lutein in the medium was found to be stable for at least 48 h. At the end of the selected incubation times, the medium was collected and cells were then washed twice with PBS containing 2 mg/mL bovine serum albumin to remove any residual lutein. Subsequently, the cells in the wells were incubated with 300 µL RIPA buffer for 5 min on ice for lysis and removal. All samples were collected into 2 mL

Eppendorf™ tubes, flushed with N_2 gas, wrapped in parafilm, and stored at -80°C until analysis. All experiments and analyses were conducted under red light.

To examine the uptake and secretion of lutein in PS, cells grown in the medium supplemented with 1% antibiotics (penicillin-streptomycin) were seeded at a density of 5 x 10^4 cells/well on collagen-coated Transwell® permeable filters (12 well plate, 0.9 cm^2 growth area, 3 µm pore size). Cells were grown for 21 d (medium changed every 2-3 d) to attain complete differentiation and monolayer integrity [4]. Alkaline phosphatase activity, an index of differentiation, was measured spectrophometrically in cell lysate, according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The cell lysate was collected using 210 µL 0.16% digitonin in 2 mM EDTA solution at 37°C and then utilized to measure protein content to reflect cell count. Trans-epithelial electrical resistance (TEER), reflective of monolayer integrity, was measured using a voltohmmeter equipped with a chopstick electrode (EVOHM2, World Precision Instruments, Sarasota, FL).

At the beginning of each PS experiment, the apical side of Caco-2 cells was washed with PBS twice and replaced with 0.5 mL lutein enriched serum-free media. The basolateral compartment was filled with 1.5 mL serum-free media. The range of the time course (0-48 h) was selected based on the data of chylomicron production and secretion to the basolateral compartment in a pilot experiment. We noted a 50% increase in apo-lipoprotein-B (apo-B) production between 6 and 16 h, but a steady state was not apparent at 36-48 h (data not shown). Apo-B was quantified using an ELISA kit (Antiobodies-Online.com, Product # ABIN612664) after chylomicron isolation using a gradient ultracentrifugation [31]. At the end of selected incubation times, both apical and basolateral media were collected. Cells remaining on the permeable filters were washed twice with PBS containing 2 mg/mL bovine serum albumin, incubated with 350 µL above and 600 µL below with 0.25% trypsin-EDTA solution for 30 min at 37°C to detach cells, and then lysed with 300 µL RIPA buffer for 5 min on ice. All samples were collected into Eppendorf™ tubes, flushed with N_2 gas, wrapped in parafilm, and stored at -80°C until analysis. All experiments and analysis were conducted under red light.

To determine the proportion of free intracellular lutein in cell lysate, which was not attached to cell membranes, additional cell samples were collected at T_{max} (identified in kinetic experiments) and combined in duplicate (all treatments for CONV and lutein in micelle for PS) or quadruplet (lutein in ethanol for PS) to ensure adequate quantification.

2.4. Lutein Extraction and Analysis

Apical and basolateral media and cell lysates (sample size of 3-4 wells) were thawed and briefly vortexed. Cell lysates were sonicated for 30 sec at room temperature. To determine the proportion of intracellular lutein, cell lysate samples were further centrifuged at 14,000 rpm for 30 min to collect cell pellets and supernatant. Lutein and β-cryptoxanthin (internal standard) in the samples were quantified according to the method of Kamil et al. [32]. Briefly, samples were extracted sequentially with FOLCH solution (chloroform/methanol: 2/1) and hexane. The organic layer was transferred, combined, and dried under N_2 gas, reconstituted in 100 µL acetone, and then analyzed by reverse phase HPLC-UV at a flow rate of 1 mL/min at 20°C on a ProntoSIL C30 column (4.6 x 150 nm, 3.0 µm, MAC-MOD Analytical, Inc., Chadds Ford, PA). Lutein and β-cryptoxanthin were monitored at 443 and 450 nm, respectively. Their concentrations were calculated using standard curves constructed with authenticated standards with concentrations ranging from 1 to 825 ng on column. The limit of detection and quantification for lutein was 0.63 and 1.0 ng on column, respectively. The recovery rate for the internal standard, calculated from 57 samples, was 71.8 ± 19.4%. The protein content of cell lysates was determined using a BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL) and was used to reflect cell count.

2.5. Statistics

All data are expressed as mean ± SD (n = 3-4 wells). The peak concentration of cell lysate and plateauing basolateral medium (C_{max}) and the time to reach C_{max} (T_{max}) were determined. The area under the curve (AUC) of apical, cell lysate, and basolateral concentration vs. time curve (0-48 h) were calculated from random complete time course curves using the linear trapezoidal integrations [33]. The differences between culture models (CONV vs PS) were analyzed using Student's t-test while the differences between doses in the culturing systems were analyzed by one-way ANOVA, followed by post hoc Tukey-Kramer honestly significant difference (HSD) test. Pearson's correlation test (r value) was performed to analyze the correlation between parameters. Simple linear regression test (r^2 value) was employed to analyze the dose-response of absorption. Three-way ANOVA was performed to test the statistical significance of dose, delivery vehicle, and culturing system. Differences with $P \leq 0.05$ were considered significant. The JMP IN 4 statistical software package (SAS Institute, Cary, NC) was used to perform all statistical analyses.

3. Results

3.1. Delivery Vehicle

The time effect on the cellular uptake of lutein and its pharmacokinetic parameters in CONV is presented in Figure 1 and Table 1. The cellular uptake of lutein in ethanol reached C_{max} at 39 ± 6 h in a non-linear fashion (Table 1). The uptake of lutein increased gradually to 24 h followed by a spike and plateau at 36 h (Figure 1A). Concurrently, there was a 27% increase in lutein concentration in the apical medium from 0-4 h, followed by a decrease and then leveling off as the cell uptake reached its C_{max}. Lutein concentrations in the cell lysate were not correlated with those in the apical medium. The uptake of lutein in micelles into the cells followed a similar trend to that of the ethanol vehicle (Figure 1B). The uptake increased up until 24 h reaching C_{max} at 42 ± 7h (Table 1). Lutein in the apical medium decreased as the

uptake by cells increased and then reached a steady state as cell uptake reached its C_{max}. Unlike lutein in ethanol, there was a negative correlation between the concentrations in the cell lysate and in the apical medium (r = -0.54, P = 0.0013).

The time effect on the cell uptake and secretion of lutein and its pharmacokinetic parameters in PS are presented in Figure 2 and Table 1. The cellular uptake of lutein in ethanol reached C_{max} at 12 ± 10.6 h (Table 1). Concurrently, lutein concentration in the apical medium decreased by 24% over 8 h without further change to 48 h (Figure 2A). The secretion of lutein in ethanol increased after lutein in the cell lysate reached C_{max} at 0.016% of dose without further change to 48 h. Although lutein concentrations in the basolateral medium were inversely correlated over time with that in the apical medium (r = -0.81, P ≤0.0001), no correlation was observed between lutein in the cell lysate and the other 2 compartments. The uptake of lutein in micelle into cells reached C_{max} at 8 h followed by a subsequent decrease towards its baseline concentration at 2 h (Figure 2B). Concurrently, lutein concentration in the apical medium decreased over time as the cell lysate reached C_{max}, followed by a decrease through 48 h. Lutein concentrations in the cell lysate were inversely correlated with that in the apical medium (r = -0.82, P = 0.001). The secretion of lutein in micelle increased in a linear manner starting at 4 h and reached a plateau at 0.15% of the dose after 36 h (Figure 2B). Similarly, lutein concentrations in the cell lysate as well as in the apical medium were correlated with that in the basolateral medium (r = 0.91, P = 0.0006 and r = -0.97, P ≤0.0001, respectively).

Table 1. Pharmacokinetics of cell lysate of Caco-2 cells cultured on CONV and PS and treated with lutein in ethanol or micelles[1,2]

	C_{max} (μg/mg protein)		T_{max} (h)		AUC (h*ng/mg protein)	
Vehicles	CONV	PS	CONV	PS	CONV	PS
Ethanol	2.8 ± 0.5	0.5 ± 0.3*	39 ± 6	12 ± 10.6*	41 ± 16	16 ± 6.7*
Micelle	13.2 ± 3.9	3.5 ± 1.5*	42 ± 7	8*	178 ± 59	130 ± 180

[1]Values are expressed as mean ± SD (n = 3 and 4 wells for PS and CONV, respectively) [2]Percentage C_{max} free in cell indicates proportion of C_{max} within cytosol and not attached to cell membranes
*Means within the same the vehicles in the same row differ, tested by Student's t-test (P ≤0.05).

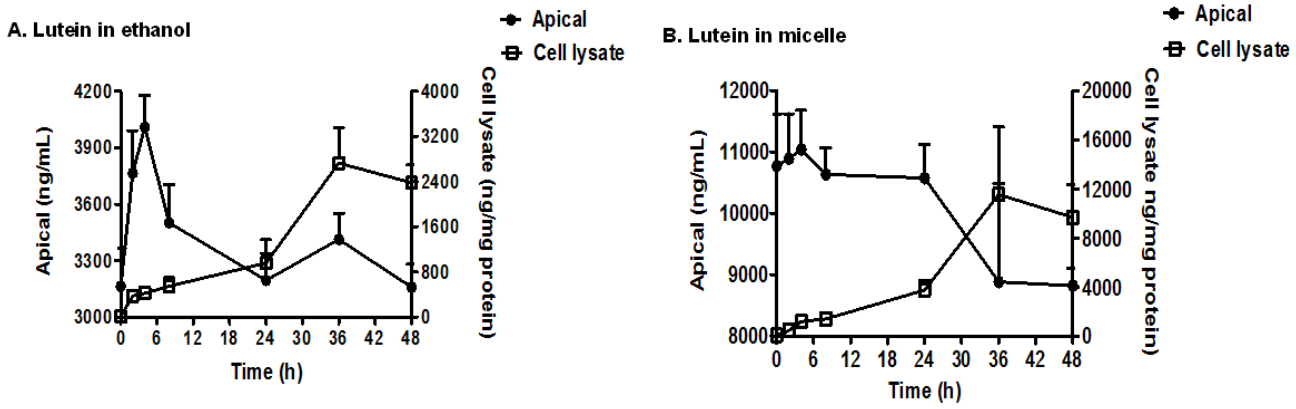

Figure 1. Time course of lutein uptake by Caco-2 cells grown on CONV and then treated with 4 μg/mL lutein in ethanol (A) or 14.6 μg/mL lutein in micelles (B). Values are expressed as mean ± SD, n = 4 wells.

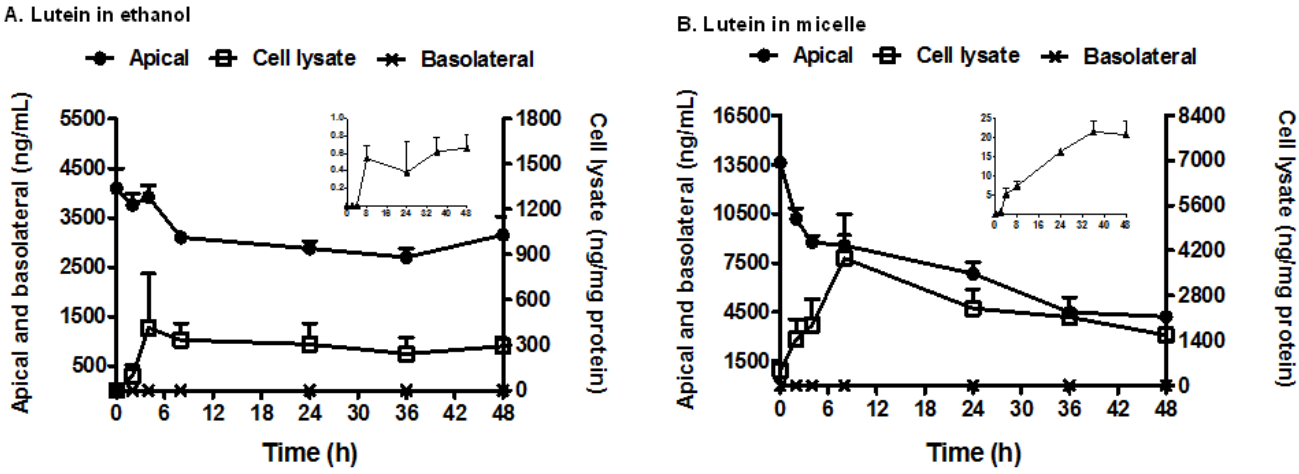

Figure 2. Time course of lutein uptake and secretion by Caco-2 cells grown on PS and then treated with 4 μg/mL lutein in ethanol (A) or 14.6 μg/mL lutein in micelles (B). Values are expressed as mean ± SD, n = 4 wells

3.2. Culture System

The effect of the culture system on lutein uptake and pharmacokinetic parameters are presented in Figure 3 and Table 1. After 2 h, the uptake of lutein in ethanol into cells in PS was 299% greater than CONV. At 24 h, the uptake in CONV was 214% greater than PS ($P \leq 0.05$, Figure 3a). Further, C_{max} for CONV was 460% greater than that for PS, T_{max} was delayed by ~27 h, and AUC was 156% greater ($P \leq 0.05$). The effect of the culture system on the uptake of lutein in micelle was similar to lutein in ethanol. At 8 h, the uptake of lutein in micelles into cells in PS was 178% greater than CONV. At 24 h, the uptake in CONV was at >81% than PS. Further, C_{max} for CONV was >277% than that for PS and T_{max} was delayed ~34 h ($P \leq 0.05$), yet the AUC did not differ. The percent of lutein uptake at T_{max} was not affected by culture system but by delivery vehicle (Table 1). As compared to micelles, the amount of lutein inside cells was >154% when it was delivered in ethanol. Interestingly, in CONV, cellular lutein concentrations were correlated between ethanol and micelle vehicles (r = 0.97, $P \leq 0.0001$), but were not correlated in the apical medium. In PS, the concentrations in the basolateral medium were correlated between 2 delivery vehicles (r = 0.75, $P \leq 0.0001$) as well as in the apical medium (r = 0.76, $P \leq 0.0001$). However, they were not correlated in the cell lysates.

The impact of the culture system on lutein absorption as the sum of cell uptake and secretion in PS (expressed as % of dose) after 36 h of incubation is presented in Figure 4. In CONV, the absorption rate for lutein in ethanol at 0.35 and 1.18 µg/mL was comparable at ~16% and increased to 44% at the 4 µg/mL (Figure 4A). Unlike the pattern noted in CONV, the absorption rate for lutein in ethanol in PS reached the highest at 1.18 µg/mL. In 2 of the 3 doses of lutein in ethanol, the absorption rate of CONV was ≥422% greater than PS (17-45% vs. 0.69-13.6%, $P \leq 0.05$), particularly at the lowest and highest doses. In CONV with lutein in micelle, the absorption rate increased in a dose-dependent fashion from 0.35 to 14.6 µg/mL ($r^2 = 0.57$, $P = 0.0156$), but the rate of the 3 lowest doses did not differ. Even though there was a dose-response relationship using PS from 0.35 to 14.6 µg/mL lutein in micelle ($r^2 = 0.86$, $P = 0.0026$), the absorption rate between the 4 doses did not differ. Unlike lutein in ethanol, only at the highest dose, the absorption rate of CONV was >319% than with PS (79.2% vs. 18.9%, $P \leq 0.05$). It is noteworthy that despite these differences, the variation in absorption between vehicles using CONV appears to be ≥252% than those using PS. Within each culture system, the absorption rate between delivery vehicles was not statistically different.

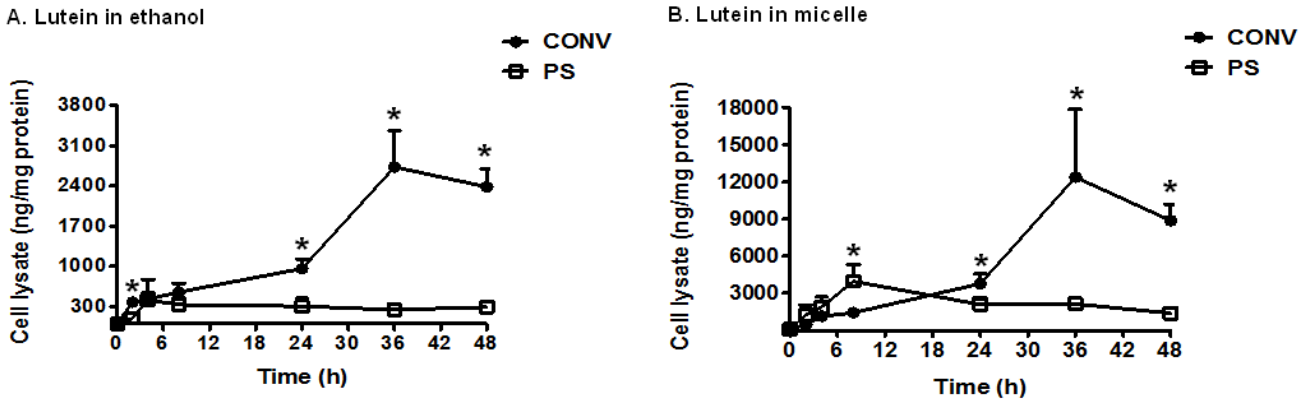

Figure 3. Time course of lutein uptake into Caco-2 cells grown on CONV and PS and then treated with 4 µg/mL lutein in ethanol or 14.6 µg/mL lutein in micelle (B). Values are expressed as mean ± SD, n = 3 and 4 wells for PS and CONV, respectively. *Means between culturing systems at the same time point differ, tested by Student's t-test ($P \leq 0.05$)

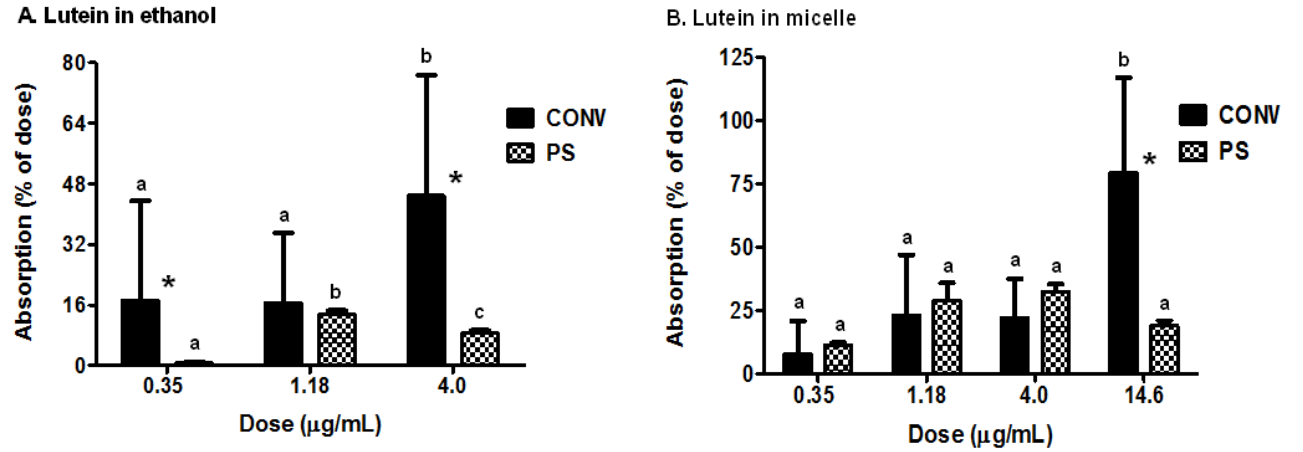

Figure 4. Lutein dose and culturing system mediate lutein absorption rate (% of dose) in Caco-2 cells treated for 36 h with lutein in ethanol (A) or micelles (B). Values are expressed as mean ± SD, n = 3 and 4 wells for PS and CONV, respectively. *Means between culture systems at the same dose differ, tested by Student's t-test ($P \leq 0.05$). [ab]Means between doses in the same system differ, assessed using one-way ANOVA, followed by Tukey's HSD test ($P \leq 0.05$)

4. Discussion

Caco-2 cells are commonly employed to model the absorption of drugs and nutrients. However, data generated from experiments using Caco-2 cells or other intestinal cell lines can be influenced by many factors. In this study, we examined the impact of the culture system (CONV vs. PS) and delivery vehicle (ethanol vs. micelle) on the uptake and secretion of the fat-soluble carotenoid lutein. When comparing C_{max} values, CONV displayed a larger efficacy of lutein uptake than PS, independent of the delivery vehicle. However, comparing the overall uptake using AUC values, the difference between the 2 systems was only significantly different when lutein was delivered in ethanol. However, compared to ethanol, micelles appeared to facilitate lutein secretion to the basolateral compartment in PS.

Intestinal uptake of carotenoids is commonly simulated in CONV along with nutrients delivered in organic solvents. Liu et al. [22] found that after 3-4 h incubation, the absorption rate of 2.3 μg/mL lutein in DMSO was 8% in Caco-2 cells grown first for 2 and 14 d. Similarly, Yi et al. [34] observed that after 24-h incubation, the absorption rate of 5 μg/mL β-carotene in a THF and DMSO mixed solvent was 3% in Caco-2 cells grown for 5 d. However, O'Sullivan et al. [20] found that after 24 h of incubation, lutein, lycopene, α-carotene or β-carotene, each at 5 μM, delivered by THF was undetectable in Caco-2 cell grown first for 21-22 d. Using ethanol as the delivery vehicle, we found that the absorption rate (17 and 45% after 36 h incubation) of 0.35-4 μg/mL lutein was higher than that previously reported. We speculate that ethanol may be a better solvent to carry lutein and potentially other fat-soluble compounds to Caco-2 cells cultured in CONV. When synthetic micelles were used as the delivery vehicle in CONV, we found that the absorption rate varied 7.8-79.2% after 36 h, a value consistent with that reported by others. Garrett et al. [16] examined the uptake of lutein, lycopene, α-carotene, and β-carotene at 0.83, 1.95, 0.93, and 1.96 μg/mL, respectively, isolated and micellized from carotenoid-rich baby foods subjected to a simulated gastrointestinal digestion was 28-46% of the dose in Caco-2 cells grown for 11-14 d. Further, Chitchumroonchokchai et al. [28] reported the absorption rate of 0.5 μg/mL lutein delivered in micelles prepared in the same manner as this study was 67%. The range of the absorption rates noted in our study and reported in the literature appears wide. While underlying factor(s) for this variation is unknown, we suspect that administered dose may account partly for the variability.

Little information is available regarding the impact of different delivery solvents on carotenoid absorption in Caco-2 cells cultured in PS. The reported absorption rate of micellized lutein in Caco-2 cells on PS range from 7-41% [28,35,36,37]. In our experiments, the percentage of lutein dose in the cell lysates ranged from 18-36% and in the basolateral medium ranged from 0-10%, values that are similar to those previously reported. However, in contrast to results from other laboratories, our kinetics data reveal a peak concentration within 8 h of the incubation [31,38]. We speculate that the incubation of 16-20 h administered in lipoprotein absorption studies [31,38] does not suffice for optimal lutein assimilation to chylomicrons and subsequent secretion. Further, differences in gradients for micelle preparation may affect lutein secretion. For example, tween-40 micelles, which are commonly used for carotenoid delivery, do not include bile salts and fatty acids required for chylomicron production. This notion is consistent with the minimal lutein observed in our study in which lutein was delivered in ethanol, a result emphasizing the importance of bile salts and phospholipids in the synthesis and secretion of chylomicrons required for lutein secretion [31,38].

Lutein uptake was greater with Caco-2 cells grown on CONV as compared to PS, suggesting the influence of differentiation on nutrient/drug transport into cells. Lutein can be taken into colonic cells grown to confluence in a degree similar to or more efficient than differentiated colonic cells expressing an enterocyte-like phenotype. Since non-differentiated Caco-2 cells do not synthesize sufficient apo-B proteins for lipoprotein assembly [6,40,41], future investigations are warranted to explore the extent of carotenoid secretion from undifferentiated colonic cells grown in PS.

There are limitations to these experiments that could be resolved by additional studies. Despite the widespread use and acceptability of the Caco-2 cells for investigating nutrient/drug absorption, this model suffers from shortcomings that limit its relevance for simulating and predicting *in vivo* bioavailability. Caco-2 cells are composed solely of absorptive cells whereas the intestinal epithelium is a conglomerate of absorptive enterocytes and other cell types such as goblet cells, endocrine cells, and microfold (M) cells. Several modifications have been made to more closely mimic the heterogeneity of the intestinal epithelium; e.g., co-culturing Caco-2 and HT29 cells introduces the presence of mucin-secreting goblet cells and provides a physiological transport barrier [42,43,44]. Co-culturing Caco-2, HT29, and Raji B cells may more closely resemble intestinal mucosa [48]. Thus, more research is needed for the development of a reliable and replicable methodology to generate data may better predict *in vivo* nutrient/drug absorption.

5. Conclusions

Cell culture models are often conducted to generate preliminary data to help elucidate *in vivo* nutrient/drug absorption. However, the interpretation and applicability of *in vitro* data can be confounded by factors such as cell line, delivery vehicle, culture system, incubation time, dose, etc. In this study, we found the delivery vehicle (micelle vs. ethanol) and culture system (CONV vs. PS) had a significant impact on lutein absorption and secretion in Caco-2 cells. The PS system allows Caco-2 cells to develop and display enterocyte phenotype and function albeit outside the context of an intact small intestine. Thus, PS should provide a more physiologically relevant system than CONV in efforts to elucidate nutrient and drug absorption. However, although Caco-2 cells grown on CONV possess the physiology of colonic cells, they take up lutein with a greater efficacy that those grown on PS and so appear to provide a preliminary but rapid tool for nutrient/drug absorption studies. The results of this study

provide practical guidelines regarding *in vitro* approaches to the study of nutrient/drug absorption.

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Abbreviations

PS, permeable support system;
CONV, conventional tissue culture system on treated plastic plates;
THF, tetrahydrofuran;
DMSO, dimetthylsulfoxide;
BC, β-cvryptoxanthin;
MO, 1-oleoyl-*rac*-glycerol (monoolein);
PC, 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine;
LC, 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysolecithin);
GDC, sodium glycodeoxycholate;
TDC, sodium taurodeoxycholate hydrate;
TC, taurocholic acid sodium salt hydrate;
OA, sodium oleate;
DMEM, Dulbecco's Modified Eagle Medium;
PBS, phosphate buffer saline;
BCA, bicinchoninic acid;
TEER, trans-epithelial electrical resistance;
Apo-B, Apo-lipoprotein-B;
C_{max}, peak concentration;
T_{max}, time to reach C_{max};
AUC, area under the curve

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