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The effects of Membrane Lipid Replacement with NTFactor® Lipids on increasing the bioavailability of three test nutrients

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ABSTRACT

Introduction: Previous studies indicated that lipids and nanostructured materials may improve the uptake of nutrients with moderate bioabsorption properties.

Objectives: This study evaluated the effects of Membrane Lipid Replacement with NTFactor® Lipids (NTFL) on bioabsorption of three poorly to moderately absorbed nutrients (quercetin, curcumin and coenzyme Q10) utilizing the Caco-2 epithelial cell permeability model.

Methods: Transfer across a Caco-2 epithelial cell layer has become a reference standard in the pharmaceutical and nutraceutical industries for in vitro prediction of in vivo human intestinal absorption and bioavailability of orally administered substances. The degree of bioabsorption of the test materials was assessed by monitoring the concentrations of the test materials on each side of the Caco-2 monolayers by liquid chromatography and mass spectroscopy (LCMS/MS analysis).

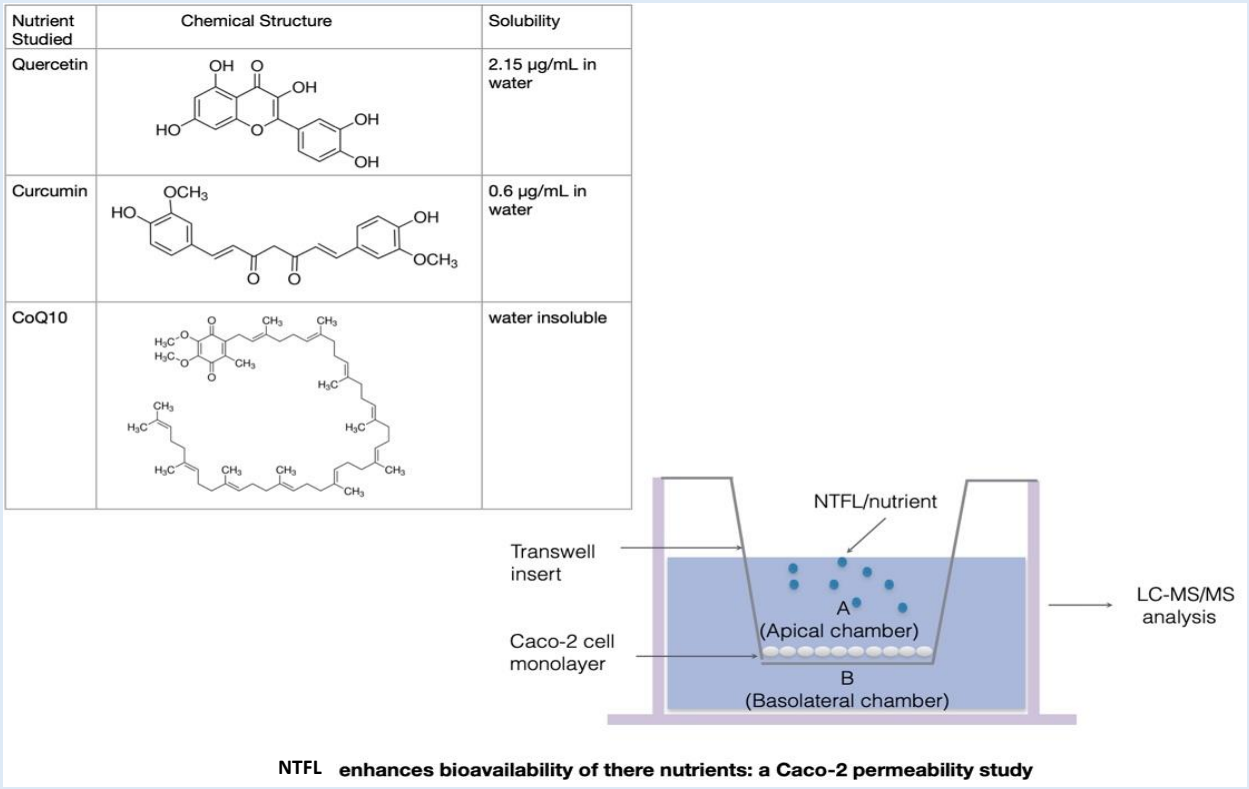
Results: When NTFL was added to each of the three test nutrients, there was increased absorption and transfer across a Caco-2 cell layer in a dose-dependent manner for the three nutrients. When compared individually, CoQ10 with NTFL showed the most significant increase in absorption (2.01-times more compared to controls without NTFL, p=0.0011) at

a concentration of NTFL of 10 mg/mL. NTFL also increased absorption and transfer across a Caco-2 cell layer of the other test nutrients, but these results did not achieve the same level of significance.

Discussion: A variety of Oral membrane lipid replacement supplements with NTFL, such as various vitamins, minerals and nutrients, have been designed to reduce fatigue, improve health conditions, and protect cellular and especially mitochondrial membranes from damage. Here we used NTFL to demonstrate improvements in absorption and bioavailability of three nutrients.

Conclusion: Using the Caco-2 bioabsorption and bioavailability in vitro model we found that NTFL could enhance absorption, bioavailability and uptake of nutrients while providing its own clinically demonstrated health benefits.

Keywords: Phospholipids, Membrane Lipid Replacement, CoQ10, curcumin, quercetin, bioavailability, absorption, permeability, Caco2, bio-uptake, bioabsorption, glycerolphospholipids, intestinal absorption



NTFL enhances bioavailability of there nutrients: a Caco-2 permeability study

INTRODUCTION

As a critical step prior to clinical studies on the effects of various nutrients on health indicators, cell-based assays are often used to provide evidence for human intestinal absorption and bioavailability of orally administered substances [1,2]. For example, *in vitro* cultured cell models for epithelial absorption and bioavailability have been developed and shown to compare well with *ex vivo* organ absorption [2-4]. In addition, various lipids have been added to oral formulations to enhance epithelial cell absorption and bioavailability [5-7]. Although other factors are involved in the physiological properties that

affect gastrointestinal transit, digestion, dissolution and epithelial absorption, the latter event is critical for the overall absorption and availability of orally consumed nutrients [6].

Several *in vitro* intestinal epithelial cell absorption models have been developed for studying absorption and metabolism of substances, especially nutrients [2,4]. One of the most widely used cell-based models for absorption and availability studies is based on the human colon cell line Caco-2 [8]. Differentiated, polarized Caco-2 cells have been used in monolayer cell cultures to mimic an epithelial cell barrier [9-11]. These cells express a wide range of transporter properties that mimic an intestinal epithelial cell layer [10,11]. The Caco-2 human intestinal monolayer uptake/bioavailability model utilizes a Caco-2 cell layer grown on a supportive membrane surface that separates two compartments where test substances can be sampled (Figure 1).

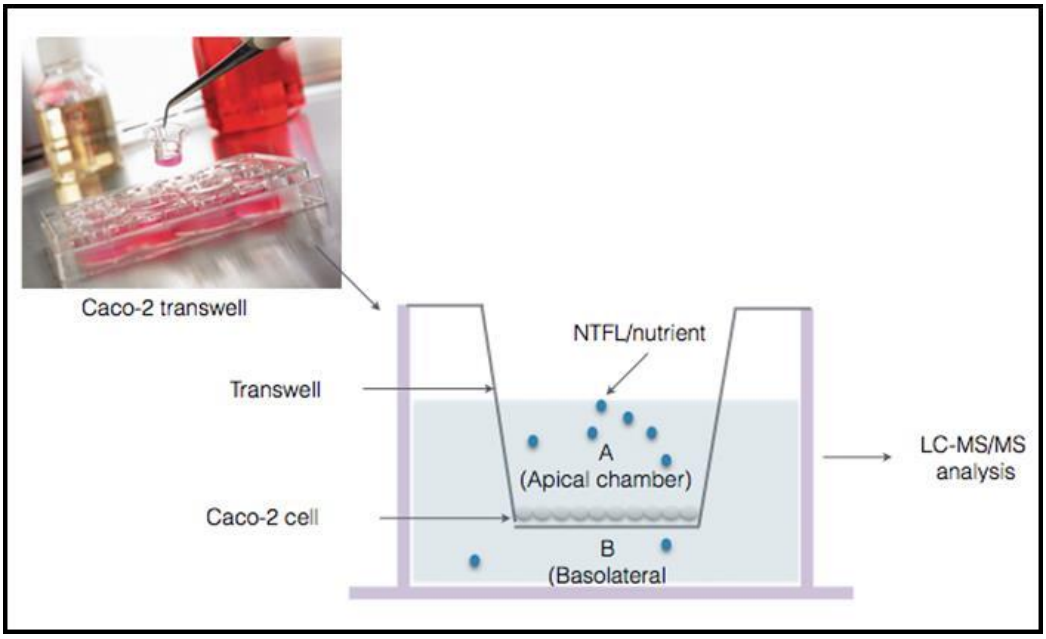


Figure 1. Schematic of Caco-2 permeability apparatus. Test nutrients were mixed with various levels of NTFL and applied to one side of the confluent monolayer of Caco-2 cells in a trans-well apparatus. The degree of biouptake of the test material was assessed by monitoring the concentration of the test material via liquid chromatography and mass spectroscopy (LCMS/MS analysis) using samples taken from the opposite side of the cell monolayer.

Caco-2 uptake studies have been used as a reference standard in pharmaceutical and nutraceutical industries for *in vitro* prediction of *in vivo* human intestinal absorption and bioavailability of orally administered substances [2,10,11]. In the drug discovery field, the U.S. Food and Drug Agency recommends that possible drug-drug interactions should be examined during drug development. The Caco-2 permeability assay has proven to be useful in predicting certain drug-drug interactions (DDIs) of orally administered drugs [12].

To enhance intestinal absorption and bioavailability lipid-based delivery systems have been developed using oils, surfactants and cosolvents [3,5,6]. Here this approach has been further developed by use of a glycerolphospholipid-based carrier that itself has health promoting properties [13-15]. In previous studies this dietary supplement, which is composed of a mixture of membrane glycerolphospholipids, known as NTFactor® Lipids (NTFL), has been used successfully in clinical and scientific studies to improve health parameters, such as fatigue, pain and other symptoms, in chronic illness

patients [14-15]. Membrane Lipid Replacement (MLR) formulas containing NTFL were shown to repair and replace damaged cellular membrane glycerolphospholipids with undamaged, unoxidized lipids, to improve function of cellular membranes, including mitochondrial membranes [14-16]. By combining the MLR glycerolphospholipids with antioxidants, MLR supplements can reduce disease-associated symptom severities, age-associated loss of function, and provide tissue and organ support [14-17].

Here the MLR supplement NTFL was used as a substitute for various oils to improve the absorption and uptake of nutrients. Using a few examples of nutrients, such as quercetin [18], curcumin [19] and coenzyme Q10 (CoQ10) [20], which all are hydrophobic phenols derived from natural plants with poor to moderate transport properties we examined the benefits of using NTFL as a carrier in the Caco-2 bioabsorption model.

MATERIALS AND METHODS

NTFL was obtained in a fine powdered form (Sample No. 20PB0807, Lot No. 193191) from Nutritional Therapeutics, Inc., Hauppauge, NY and was used to assess whether NTFL could improve the bioabsorption of nutrients that are otherwise moderately or poorly absorbed. Three nutrients were selected for this study: CoQ10, quercetin and curcumin [18-20]. They were all obtained from Sigma-Aldrich. The aqueous solubilities and chemical structures of the three nutrients are shown in Figure 2.

The selected test nutrients were mixed with the NTFL phospholipid formula and applied to one side of a confluent monolayer of Caco-2 cells in a transwell apparatus (Figure 1). Three concentration levels of NTFL were evaluated: 0.1% (1 mg/mL), 0.25% (2.5 mg/mL) and 1% (10 mg/mL). The degree of bio-uptake of the test material was then assessed by monitoring the concentration of the test material on the opposite side of the monolayer using LCMS/MS analysis. A schematic of the study design is shown in Figure 1.

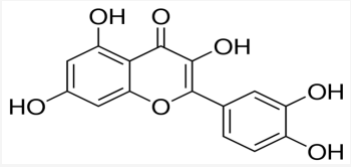
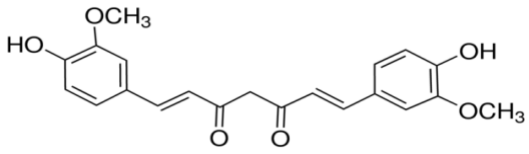
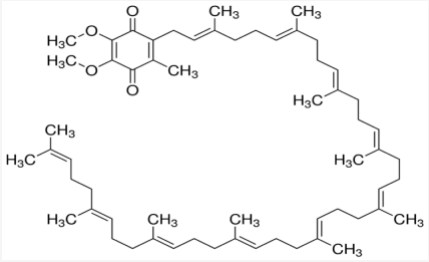
Name	Chemical Structure	Solubility
Quercetin		2.15 μg/mL in water
Curcumin		0.6 μg/mL in water
CoQ10		water insoluble

Figure 2. Chemical structures and aqueous solubilities of the test nutrients used in the study.

Preparation of solutions: Three concentration levels of NTFL in cell medium containing 0.1% ethanol were prepared. Quercetin, curcumin and CoQ10 were then mixed at various concentration levels of NTFL in cell medium at final concentrations of 20 µM, 25 µM, and 10 µM, respectively, according to the following protocol:

For the preparation of 1% NTFL (10 mg/mL) NTFL in cell media containing 0.1% ethanol was added to cell medium at ratio of 1:1000 to obtain a cell medium/0.1% ethanol solution. NTFL powder was added into cell medium/0.1% ethanol at 1% weight/volume ratio (or 1 g in 100 ml of cell media/0.1% ethanol). The mixture was ultrasonicated for 25 min at 20 KHz and subsequently filtered using a 0.2 µM filter. This resulted in a homogeneous, clear solution by visual inspection. Similarly, for the preparation of NTFL at 0.25% (2.5 mg/mL) and 0.1% (1 mg/mL) in cell medium/0.1% ethanol, NTFL was added as described above to cell media at 1:4 and 1:10 dilutions of 1% NTFL mixture with cell medium/0.1% ethanol, respectively. The mixtures were ultrasonicated for 25 min at 20 KHz. A 0.2 µM filter was subsequently used to filter the solution, resulting in a homogeneous, clear solution by visual inspection.

Caco-2 cell cultures and trans-well methods: Caco-2 human intestinal cells were grown in tissue culture flasks containing Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose supplemented with 10% fetal calf serum (FCS) with 1% non-essential amino acids (NEAA) and 0.01% gentamycin in an atmosphere consisting of 90% relative humidity with 10 % CO_2 at 37°C (growth medium). Each monolayer was treated with Hank's Balanced Salt Solution (HBSS) containing trypsin, and released cells were resuspended in fresh growth medium. The cell suspensions were applied to wells of Millipore Millicell-24 trans-well cell culture plates containing inserts with 0.4 µm polycarbonate filters. The Caco-2 cells were plated at a density of 6×10^4 cells/cm^2 on the filters in the 24-well trans-well plates and grown in DMEM containing FCS at 37°C, as described above. The cells were allowed to grow and differentiate for 21 days. The growth medium was changed in each well at 2-day intervals.

Caco-2 permeability and transport were investigated in the apical-to-basolateral direction (A→B) at 37°C (n = 3 for each concentration tested). The Caco-2 monolayers were washed with HBSS. For apical to basolateral (A→B) permeability, the test agent was added to the apical (A) side. The amount of permeation was determined from aliquots obtained from the basolateral (B) side of each trans-well. The apical (A) buffer (1.98 g/L glucose in 10 mM 4-2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1x HBSS was at pH 6.5. The B-side transport was the same, but at a pH of 7.4.

Trans-wells with Caco-2 monolayers were incubated with the above buffers for 2 h, and the donor and receiver side buffers were removed for analysis by LC-MS/MS using propranolol as an analytical internal standard. To verify that the Caco-2 cell monolayers were properly formed and thus maintained an appropriate barrier, the A-side buffer additionally contained 100 µM Lucifer Yellow dye in the transport buffer (1.98 g/L glucose in 10 mM HEPES plus 1x HBSS pH 6.5), and the amount of permeation was determined by sampling the basolateral (B) side buffer after 2 h. Aliquots of the basolateral (B) side buffers were analyzed by fluorescence to determine the possible leakage of the impermeable dye Lucifer Yellow into the basolateral (B) chamber. Those samples containing Lucifer Yellow were discarded and not included in the transport analyses.

Talinolol and Warfarin [21, 22] were used as low-permeability and high-permeability quality control substances, respectively, in order to test the trans-well transport system. For the Caco-2 permeability experimental conditions, apical to basolateral (A→B)

permeability (P_{app}) values using 20 µM quercetin, 25 µM curcumin, or 10 µM CoQ10 were mixed at three different NTFL concentration levels, and transport values were determined after incubation in trans-wells with Caco-2 monolayers for 2 h by multidimensional liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis [23].

LC-MS/MS analysis: Samples were analyzed by LC-MS/MS using a SCIEX 6500 QTRAP mass spectrometer coupled with a HPLC and a Shimadzu SIL30ACMP, all controlled by MultiQuant software. After separation on a C18 reverse phase HPLC column (Acquity HSS T3 C18 column, 1.8 µM, 2.1x50mm) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode.

Permeability data can be expressed as permeability (P_{app}), where dQ/dt is rate of permeation, C_0 is initial concentration of test agent, and A is the area of monolayer (0.7 cm^2). Permeability data are thus expressed as:

$$P_{app} = \frac{dQ/dt}{C_0 A}$$

The experimental conditions of Caco-2 permeability studies are summarized in Table 1, the control permeability study.

Table 1. Control study of poorly and highly absorbed drugs using the Caco-2 absorption model.

Test Article	Test Conc.	Assay Duration (h)	Mean A→B P_{app} (10^{-6} cm s^{-1})	Comment
Talinolol	10 µM	2	0.19	Low permeability control
Warfarin	10 µM	2	115.36	High permeability control

Statistical Methods: The observed data for the P_{app} determinations for each NTFL dose concentration were collected for analysis. For each test nutrient, a one-way ANOVA was performed, followed by a Dunnett's test (for multiple comparisons to the control group) [24]. A plot of the mean responses and the 95% confidence limits versus dose levels were constructed for each nutrient.

For analysis of absorption of all three nutrients by NTFL collectively, a two-way analysis of variances was applied. Each replicate was treated as an independent observation at each dose level. The covariates included nutrient, dose level and an interaction term between nutrient and dose level. The interaction term was included to assess a statistically significance difference in the means among the combination of nutrient and dose level strata. Thus, if the interaction effect is present (p-value < 0.05), the impact of dose level to the response depends on the nutrient, and vice versa. The estimated marginal mean responses and the 95% confidence limits at each dose level were obtained when the nutrient was adjusted in the model. A plot of the mean responses and the 95% confidence limits versus dose level was constructed.

RESULTS

To test the reliability of the Caco-2 test system a control study was conducted. Table 1 shows the Caco-2 permeability control study**.** The results indicated that the trans-well permeability system works as expected during the 2 h assay. The low-permeability Talinolol control showed a P_{aap} value of <0.5, and the high permeability Warfarin control showed a P_{app} value of >100, as expected. Tables 2 and 3 presents the analyses of the

permeability data based on the data from three observations for each nutrient and the different NTFL dose levels. For the CoQ10 nutrient, there was a statistically significant difference in means of responses for the different dose levels (p=0.002). For each nutrient, the plots of the mean response versus dose level and the 95% confidence limits for the mean are shown in Figure 3. For the CoQ10 nutrient, there is a statistically significant dose level effect with a p-value of 0.002.

As shown in Table 2, the estimated marginal means of P_{app} and 95% confidence intervals from the ANOVA model without the interaction term were 0.96 [95% CI (0.77, 1.15)] for the Control, 1.04 (0.84,1.23) for the 0.1% (1 mg/mL) concentration of NTFL, 1.28 (1.09, 1.47) for 0.25% (25 mg/mL) NTFL, and 1.63 (1.43, 1.82) for the 1% (10 mg/mL) NTFL level. A graph of the marginal mean response for the control and three different NTFL dose levels is shown in Figure 3. For each dose level, the data represents the mean, and the vertical bar represents the 95% confidence level of the mean. The graphs show the increasing trends in the marginal means of responses across the different dose levels.

Table 2. Analyses of permeability assays at different concentrations of NTFL (N=3).

Nutrient	NTFL Dose	N	Mean	Std Dev	Minimum	Maximum	Absorption Increase
CoQ10	A: Control (0%)	3	0.90	0.10	0.81	1.00	-
	B: 0.1% (1 mg/mL)	3	1.00	0.20	0.83	1.22	1.11
	C: 0.25% (2.5 mg/mL)	3	1.17	0.23	1.01	1.43	1.30
	D: 1.0% (10 mg/mL)	3	1.81	0.23	1.64	2.07	2.01
Curcumin	A: Control (0%)	3	0.56	0.06	0.51	0.62	-
	B: 0.1% (1 mg/mL)	3	0.58	0.12	0.49	0.71	1.03
	C: 0.25% (2.5 mg/mL)	3	0.96	0.22	0.72	1.14	1.71
	D: 1.0% (10 mg/mL)	3	1.25	0.68	0.78	2.03	2.23
Quercetin	A: Control (0%)	3	1.42	0.13	1.29	1.55	-
	B: 0.1% (1 mg/mL)	3	1.53	0.22	1.29	1.73	1.08
	C: 0.25% (2.5 mg/mL)	3	1.71	0.42	1.25	2.08	1.20
	D: 1.0% (10 mg/mL)	3	1.83	0.35	1.42	2.05	1.29

Table 3. The estimated marginal means of P_{app} and 95% confidence intervals from the one-way ANOVA model.

Nutrient	NTFL Dose	P-value for Test Dose Effect	Estimated Mean of P_{app} and 95% CL	Estimated Mean Difference from Control and 95% CL	Adjusted p-value of Comparison to Control by Dunnett's Method
Quercetin	A: Control (0%)	0.413	1.42 (1.01, 1.82)		
	B:0.1% (1 mg/mL)		1.53 (1.13, 1.94)	0.12 (-0.6, 0.83)	0.93401
	C: 0.25% (2.5 mg/mL)		1.71 (1.3, 2.11)	0.29 (-0.42, 1)	0.539549
	D: 1% (10 mg/mL)		1.83 (1.42, 2.23)	0.41 (-0.3, 1.12)	0.296371
Curcumin	A: Control (0%)	0.137	0.56 (0.08, 1.04)		
	B: 0.1% (1 mg/mL)		0.58 (0.1, 1.06)	0.02 (-0.84, 0.88)	0.999762
	C: 0.25% (2.5 mg/mL)		0.96 (0.48, 1.44)	0.4 (-0.46, 1.26)	0.439218
	D: 1% (10 mg/mL)		1.25 (0.76, 1.73)	0.69 (-0.17, 1.54)	0.115967
CoQ10	A: Control (0%)	**0.002**	0.9 (0.65, 1.16)		
	B: 0.1% (1 mg/mL)		1 (0.75, 1.26)	0.1 (-0.36, 0.56)	0.862942
	C: 0.25% (2.5 mg/mL)		1.17 (0.92, 1.43)	0.27 (-0.19, 0.73)	0.276621
	D: 1% (10 mg/mL)		1.81 (1.56, 2.07)	0.91 (0.45, 1.37)	**0.001125**

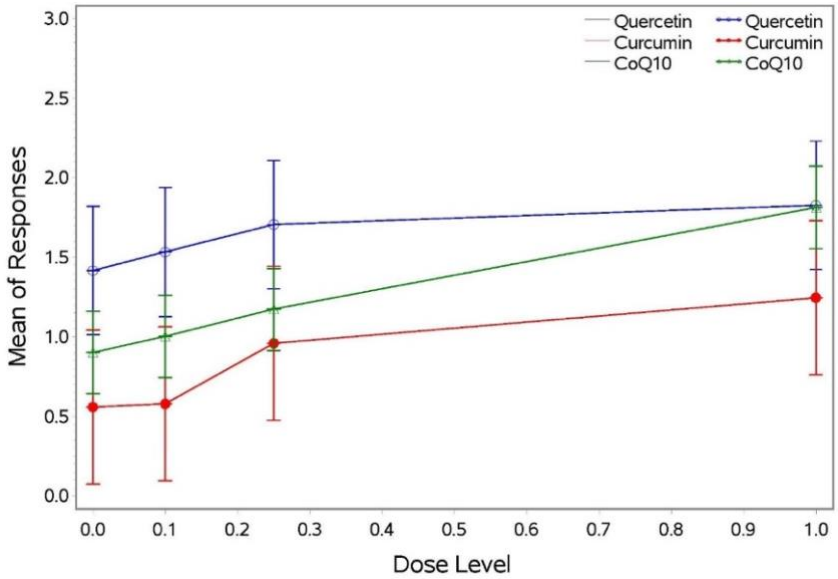


Figure 3. A plot of the mean responses and the 95% confidence limits versus dose level was constructed for each nutrient. The dot represents the mean and the vertical bar represents the 95% confidence limit of the mean. The means of responses were connected across the dose level. For the CoQ10 nutrient, there is a statistically significant dose level effect with a p-value of 0.002. The estimated mean difference between the dose level of 1% (10 mg/mL) NTFL and Control is 0.91 (95%CI; 0.45, 1.37) with a p-value of 0.001125 after Dunnett's multiple comparison method was applied. However, based on the observed data, we were unable to detect a statistically significant dose effect for Quercetin and Curcumin, separately.

DISCUSSION

We found here that NTFL improved permeability/absorption over baseline values of the three nutrients tested in a dose-dependent manner. For example, Caco-2 bioabsorption studies of CoQ10 with 1% NFTL indicated that estimated marginal means of P_{app} CoQ10 transport was increased 2.01-times over controls without NTFL (p= 0.0011). Although there were positive dose-dependent responses of curcumin and quercetin absorption with NTFL, these did not reach the same level of significance as seen with CoQ10. The lipophilicity of the three nutrients likely contributed to the effects seen with added NTFL. CoQ10, clearly the most lipophilic of the three nutrients, was successfully absorbed with NTFL, whereas quercetin, the least lipophilic, was the least well absorbed with added NTFL.

MLR with oral glycerolphospholipids has been used successfully in multiple clinical studies in humans to repair damage to cellular membranes and reduce symptom severity [13-17]. The MLR supplement/ingredient used in these studies, NTFactor® Lipids, contains several types of glycerolphospholipids plus fructooligosaccahrides to protect the phospholipids from disruption, degradation and oxidation in the gut and antioxidants to protect against oxidative damage. This MLR formulation has been shown to improve health outcomes in chronic illness patients with no evidence of any adverse events [13-17]. The membrane glycerolphospholipids in MLR supplements are quickly and almost completely absorbed and transported into tissues and cells without excessive degradation [14,15]. There the undamaged, replacement membrane phospholipids can exchange with damaged membrane phospholipids, resulting in replacement of the damaged molecules. The NTFactor® Lipids glycerolphospholipids also provide important precursors for specific membrane molecules, such as mitochondrial cardiolipin [14-17]. Thus the use of MLR supplements like NTFactor® Lipids glycerolphospholipids has additional benefits beyond helping to transport nutrients from the gastrointestinal system into tissues and cells.

This study demonstrated that the bioavailability of nutrients when combined with NTFL glyerophospholipid formulations are likely to result in increased absorption and transport through intestinal epithelial cells compared with the nutrients without added NTFL, suggesting strongly that NTFL promotes nutrient bioavailability when taken orally. In addition, as noted above, NTFL formulations on their own have significant health benefits in both animal and human clinical studies [13-15]. Therefore, combination nutrient formulas with NTFL as an additive ingredient should provide not only the enhanced absorption of nutrients but also the potential ability of NTFL to provide support for cellular membranes and mitochondrial function [15-17].

List of Abbreviations: Caco-2, human cancer adenocarcinoma cell line; CoQ10, co-enzyme Q10; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HEPES, 4-2-Hydroxyethyl)-1-piperazineethanesulfonic acid; MLR, Membrane Lipid Replacement; NEAA, non-essential amino acids; NTFL, NTFactor® Lipids.

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Author Contributions: R. Setterini, concept, design, management and initial draft of the manuscript; J. Ji, supervision of laboratory experiments; Z.P. Shields,

experimental assistance; T. Shirvani, technical advice and manuscript review; C.E. McLaren, statistical assistance and manuscript review; G.L. Nicolson, technical advice and final manuscript production.

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