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Acute and chronic effects of some dietary bioactive compounds on folic acid uptake and on the expression of folic acid transporters by the human trophoblast cell line BeWo

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Abstract

Folic acid (FA) is a vitamin that acts as a coenzyme in the biosynthesis of purine and pyrimidine precursors of nucleic acids, which are critically important during pregnancy. Our group has previously shown that both reduced folate carrier (RFC1) and folate receptor α (FRα) seem to be involved in the uptake of [^{3}H]folic acid ([^{3}H]FA) by a human trophoblast cell line (BeWo) and by human primary cultured cytotrophoblasts. Our aim was to study the interaction between FA and some nutrients/bioactive substances. For this, we tested the acute and chronic effects of some dietary compounds on [^{3}H]FA apical uptake and on the expression of both RFC1 and FRα mRNA in BeWo cells. Our results show that [^{3}H]FA uptake was significantly reduced by acute exposure to epicatechin, isoxanthohumol (1–400 μM) or theophylline (0.1–100 μM); isoxanthohumol seemed to act as a competitive inhibitor, whereas epicatechin and theophylline caused an increase in both K_m and V_{max}. On the other hand, [^{3}H]FA uptake was significantly increased by chronic exposure to xanthohumol, quercetin or isoxanthohumol (0.1–10 μM), and this increase does not seem to result from changes in the level of RFC1 or FRα gene expression. Moreover, [^{3}H]FA uptake was significantly reduced by chronic exposure to ethanol (0.01%). This reduction seems to be, at least in part, due to a reduction in FRα expression. These results are compatible with an association between a deficient FA supply to the placenta/fetus and ethanol toxicity in pregnancy.

Keywords: Folic acid; Reduced folate carrier; Folate receptor; Ethanol; Polyphenols; Methylxanthines

1. Introduction

Folic acid (pteroylglutamate; FA) is the parent structure of a large family of B-vitamin coenzymes known as folates. Various coenzymes of FA facilitate the transfer of one-carbon units in reactions leading to the synthesis of methionine, thymidine and purine nucleotides, which are crucial in processes such as cell division. For this reason, FA is critically important for normal fetal development during pregnancy, as demonstrated by the well-established association between maternal FA deficiency and pregnancy complications such as preeclampsia [1,2] and a higher incidence of fetal neural tube defects (NTDs) [1]. Periconceptional supplementation with FA is now widely accepted as a strategy for reducing the risk of NTDs [3–5].

Knowing that FA in food or supplementation is ingested together with other nutrients and bioactive substances and that the developing fetus obtains FA from the maternal blood through the placenta, it is of major importance to study the interactions between FA and those nutrients/substances that may improve or inhibit FA absorption at the placental level.

So, our aim was to determine the effect of bioactive substances present in alcoholic and nonalcoholic beverages on FA placental uptake by testing the acute and chronic effects of these substances on [^{3}H]folic acid ([^{3}H]FA) uptake by BeWo cells.

One of the substances tested was ethanol because it is the most frequently used drug worldwide [6] and its consumption is not uncommon during pregnancy. For example, in the United States, between the years 2003 and 2004, 11% of pregnant women aged 15–44 years reported alcohol use and 4.5% reported binge drinking during the prior month. Nevertheless, heavy alcohol use was relatively rare (0.5%) among pregnant women [7,8]. Among the wide variety of

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medical problems caused by alcohol abuse, chronic alcoholism has been known for a long time to cause deficiency in several nutrients, including the vitamin FA [6,9–11]. Moreover, alcohol abuse during pregnancy produces permanent fetal brain damage and a wide variety of manifestations, known as fetal alcohol spectrum disorder (FASD).

Other substances tested were some polyphenols (catechin, chrysin, epicatechin, epigallocatechin-3-gallate, isoxanthohumol, myricetin, quercetin, resveratrol, rutin and xanthohumol) present in alcoholic (e.g., red wine and beer) and nonalcoholic beverages (e.g., green tea), as well as some methylxanthines (caffeine and theophylline) present in drinks such as coffee and tea.

The human placenta expresses reduced folate carrier (RFC1) [12–14], folate receptor α (FRα) [15–17] and folate receptor β (FRβ) [18–20]. Although both the FR isoforms are detectable in whole placental tissue, it is only the FRα isoform that is selectively expressed in normal trophoblast cells and choriocarcinoma cells. FRβ isoform is probably present in the maternal decidua, which is closely associated with fetal tissue after delivery [21–23].

It is widely accepted that both RFC1 and FRα may have a role in the process of FA transport from maternal circulation to fetal circulation. Previous work by our group has suggested that, at physiological pH, there is involvement of both RFC1 and FRα in FA uptake by the human trophoblast cell line BeWo [24] and by human primary cultured cytotrophoblasts [25]. So, we also determined, by reverse transcription–polymerase chain reaction (RT-PCR), the chronic effects of dietary substances on the expression of both RFC1 and FRα mRNA.

2. Materials and methods

2.1. BeWo cell culture

The BeWo cell line was obtained from the American Type Culture Collection (ATCC CCL-98; Rockville, MD, USA) and was used between passage numbers 7 and 39. Cells were maintained in a humidified atmosphere of 5% CO_2–95% air and grown in Ham's F12K medium containing 2.5 g/L sodium bicarbonate, 10% heat-inactivated fetal calf serum and 1% antibiotic/antimycotic solution. Culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37°C), split 1:2–1:3 and subcultured in plastic culture dishes (21 cm^2, ∅ 60 mm; TPP, Trasadingen, Switzerland). For transport studies, BeWo cells were seeded on collagen-coated 24-well plastic cell culture clusters (2 cm^2, ∅ 16 mm; TPP) and used after 3–5 days in culture (90–100% confluence). At this moment, each square centimeter contained about 60 μg of cell protein.

2.2. Transport studies

Transport experiments were performed in a buffer with the following composition (in mM): 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 12.5 HEPES (*N*-2-hydroxyethylpiperazine-*N*′ -2-ethanesulfonic acid)–NaOH, 12.5 MES (2-[*N*-morpholino]ethanesulfonic acid), 1.2 $MgSO_4$, 1.2 $CaCl_2$ and 5.6 D(+)glucose (pH 7.5). Initially, the culture medium was aspirated, and the cells were washed with buffer at 37°C; then cell monolayers were preincubated for 20 min in buffer at 37°C. Uptake was initiated by the addition of 0.2 ml of buffer at 37°C containing 20 nM [^{3}H]FA (except in experiments for the determination of the kinetics of [^{3}H]FA uptake). Incubation was stopped after 6 min by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml of ice-cold buffer. The cells were then solubilized with 0.3 ml of 0.1% vol/vol Triton X-100 (in 5 mM Tris–HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting.

2.3. Acute and chronic treatment of cells

The concentrations of compounds used to test both acutely and chronically were chosen based on previous works by our group [26–28].

The acute effect of compounds on [^{3}H]FA uptake by BeWo cells was tested by preincubating (20 min) and incubating cells with [^{3}H]FA (20 nM; 6 min) in the presence of the compounds to be tested.

The chronic effect of compounds on [^{3}H]FA uptake by BeWo cells was tested by cultivating 3-day-old cell cultures in culture medium containing the compounds to be tested (ethanol, xanthohumol, catechin, epicatechin, *iso*-xanthohumol, resveratrol, quercetin, myricetin, epigallocatechin-3-gallate, chrysin, rutin, caffeine and theophylline). The medium was renewed daily, and transport experiments were performed after 48 h. The transport experiments were identical to the experiments described above, except that there was no preincubation period and cells were incubated with [^{3}H] FA in the absence of drugs.

To test the specificity of the acute and chronic effects of these compounds on [^{3}H]FA uptake by BeWo cells, these compounds were also tested in relation to [^{3}H]thiamine (100 nM, 3 min of incubation) and [^{14}C]alanine (250 nM, 6 min of incubation) uptake by BeWo cells.

2.4. Assessment of cell viability

The effect of dietary compounds on BeWo cell viability was determined by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay [29].

To test whether the compounds that had an acute effect (26 min) on [^{3}H]FA uptake affected cellular viability, BeWo cells were incubated for 3 h at 37°C in 500 μl of the culture medium with 0.5 mg/ml MTT solution. In the last 26 min of this period, the compounds to be tested were added.

To test whether the compounds that had a chronic effect (48 h) on [^{3}H]FA uptake affected cellular viability, BeWo cells where chronically treated with the compounds described above. After 45 h of treatment, 50 μl of MTT

solution (5 mg/ml) was added to each well. The cells were then further incubated for 3 h at 37°C.

MTT solution was removed after the 3-h incubation period, and the cells were lysed by the addition of 200 μl of dimethylsulfoxide (DMSO) followed by plate shaking for 10 min at room temperature. Optical density for the solutions in each well was determined at both 540 and 660 nm. Optical density at 660 nm corresponds to unspecific light absorption and was subtracted from the OD at 540 nm to give the OD value specific to formazan crystals derived from MTT cleavage.

2.5. RNA extraction and RT-PCR

Total RNA was extracted from chronically treated BeWo cells (see above) by the method of Chomczynski and Sacchi [30]. Before RNA extraction, cell culture medium was aspirated, and the cells were resuspended in buffer (with the composition shown above) and centrifuged at 3000×*g* for 5 min to completely remove the culture medium. RNA was extracted with phenol–chloroform, precipitated with ethanol and dissolved in water. For cDNA synthesis, 5 μg of RNA thus prepared was incubated at 45°C for 1 h in a total volume of 20 μl with 200 U of M-MuLV reverse transcriptase RNase H^- (Bioron GmbH, Ludwigshafen, Germany) in 10 μM random hexamers (Sigma, St. Louis, MO, USA), 0.375 mM dNTP (Bioron GmbH), 3 mM $MgCl_2$, 100 mM KCl, 50 mM Tris–HCl (pH 8.3; 25°C), 10 mM dithiothreitol and 40 U of RNase inhibitor (RNaseOUT; Invitrogen Corporation, Carlsbad, CA, USA). Following heat inactivation of proteins (10 min at 95°C) and the addition of 5 μl of 0.5 mg/ml DNase-free RNase A (Sigma) to 10 mM Tris–HCl (pH 8.0) and 50% vol/vol glycerol, cDNA was incubated at 37°C for 30 min to degrade unreacted RNA. Using 4 μl of this preparation, PCR was performed. The PCR mixture (50 μl) contained 0.5 μM per primer, 0.2 mM per dNTP, 2.3 mM $MgCl_2$ and 2 U of DFS-Taq DNA polymerase (Bioron GmbH).

Six microliters of each individual PCR reaction was then run on 1.6% agarose gel and visualized with an ultraviolet transilluminator (Vilber Lourmat, Marne La Vallée, France) using ethidium bromide staining, a COHU CCD camera and appropriate filters for UV light. Band intensity was determined by Gel-Pro Analyzer version 3.1 (Copyright Media Cybernetics, 1993–1997).

The primer pairs used for amplification and the predicted sizes of PCR products were: 5′-AGA TAC GGC CAG GGG AGA GCT TCA T-3′ (forward) and 5′-GTA GGA GGA ATA GGC GAT GCG CGC-3′ (reverse) for human RFC1 (299 bp); 5′-CTC TAC GAG TGC TCC CCC AAC TTG-3′ (forward) and 5′-GTC AGC TGA GCA GCC ACA GCA GCA-3′ (reverse) for human FRα (460 bp); 5′-ACT GGC GTC TTC ACC ACC AT-3′ (forward) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 682 bp). The thermocycling conditions for RFC1 were: 95°C for 5 min (1 cycle); 94°C for 60 s, 60°C for 90 s, 72°C for 90 s (30 cycles); and 72°C for 10 min (1 cycle). For FRα, thermocycling conditions were 94°C for 5 min (1 cycle); 94°C for 60 s, 65°C for 90 s, 72°C for 90 s (30 cycles); and 72°C for 10 min (1 cycle).

2.6. Protein determination

The protein content of cell monolayers was determined as described by Bradford [31] using human serum albumin as standard.

2.7. Calculations and statistics

For analysis of the saturation curve, the parameters of Michaelis–Menten equation were fitted to experimental data with nonlinear regression analysis using a computer-assisted method [32].

For semiquantitative analysis of RT-PCR bands, the relative density of each band was calculated against the corresponding GAPDH band density.

Arithmetic means are given with S.E.M. The statistical significance of the difference between various groups was evaluated by one-way analysis of variance followed by Bonferroni test. For comparison between two groups, Student's *t* test was used. Differences were considered to be significant when $P<.05$.

The value of *n* indicates the number of replicates of at least two different experiments.

2.8. Materials

The materials used were as follows: [^{14}C]alanine (L-[U-^{14}C]alanine; specific activity, 154 mCi/mmol), [^{3}H]FA ([3′,5′,7,9-^{3}H]folic acid potassium salt; specific activity, 21.0 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK); [^{3}H]thiamine ([^{3}H(G)]thiamine hydrochloride; specific activity, 10 Ci/mmol; Biotrend Chemikalien GmbH, Cologne, Germany); (+)catechin hydrate, chrysin, collagen type I, epicatechin, (−)epigallocatechin-3-gallate, folate, Ham's F12 K (nutrient mixture F12-Ham Kaighn's modification), HEPES, MES hydrate, myricetin, quercetin dihydrate, resveratrol, rutin, theophylline, trypsin–EDTA solution, (Sigma); DMSO, Triton X-100, Tris [tris-(hydroxymethyl)-aminomethane hydrochloride; Merck, Darmstadt, Germany]; fetal calf serum (Invitrogen Corporation); caffeine (BDH Laboratory Chemicals Ltd., Poole, England).

Xanthohumol and isoxanthohumol were kindly donated by Eng. José M. Machado Cruz [Instituto de Bebidas e Saúde (iBeSa), S. Mamede Infesta, Portugal].

When the drugs to be tested were dissolved in ethanol or DMSO, the final concentration of these solvents was 1% in the buffer or 0.1% in the culture medium for acute or chronic treatment, respectively. Controls for these drugs were run in the presence of the solvent.

3. Results

Previous experiments from our group have shown that [^{3}H]FA uptake by BeWo cells was linear with time up to the sixth minute of incubation [24]. So, a 6-min incubation

period was used in the present experiments in order to study the effect of dietary bioactive compounds on [^{3}H]FA uptake.

3.1. Effect of dietary bioactive compounds on [^{3}H]FA uptake

3.1.1. Acute effect

The acute (26 min) effect of different concentrations of ethanol (0.1–5% vol/vol) on [^{3}H]FA uptake by BeWo cells was tested. None of the concentrations of ethanol had a significant effect on vitamin uptake (results not shown).

The acute effect of some polyphenols (catechin, chrysin, epicatechin, epigallocatechin-3-gallate, isoxanthohumol, myricetin, quercetin, resveratrol, rutin and xanthohumol) and two methylxanthines (caffeine and theophylline) was also determined. Of these compounds, epicatechin (100 and 400 μM), isoxanthohumol (10–400 μM) and theophylline (10 and 100 μM) inhibited [^{3}H]FA apical uptake by BeWo cells (Fig. 1). Interestingly, the maximum effect was quantitatively similar for the three compounds (around 30% inhibition). None of the other compounds tested (all in concentrations ranging from 0.1 to 100 μM) had any acute effect on [^{3}H]FA transport in this cell line (data not shown).

3.1.2. Chronic effect

The chronic (48 h) effect of 0.01% and 0.1% (vol/vol) ethanol on [^{3}H]FA uptake by BeWo cells was investigated next. In contrast to what was seen with the acute exposure of BeWo cells to this agent, we verified that chronic exposure to the lowest concentration of ethanol inhibited [^{3}H]FA uptake to about 65% of control (Fig. 2).

The chronic effect of polyphenols and methylxanthines was also investigated. Of all the compounds tested, only three had a significant effect on [^{3}H]FA apical uptake by BeWo cells (Fig. 3). These compounds were xanthohumol, quercetin (1 and 10 μM) and isoxanthohumol (10 μM), which caused a significant increase in [^{3}H]FA uptake.

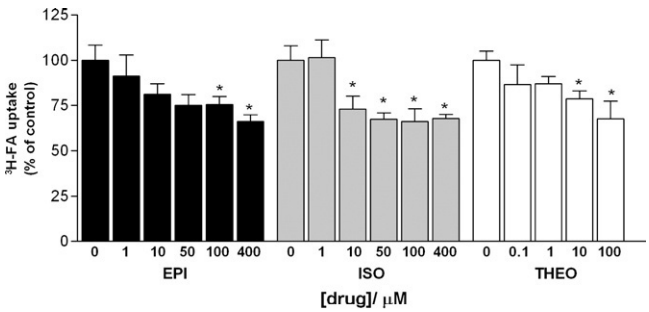

Fig. 1. Effect of the acute exposure of BeWo cells to different concentrations of the polyphenols epicatechin (EPI; $n=6$–11) and isoxanthohumol (ISO; $n=4$–7) and the methylxanthine theophylline (THEO; $n=4$–6) on [^{3}H]FA apical uptake. BeWo cells were preincubated for 20 min and then incubated with 20 nM [^{3}H]FA for 6 min at 37°C in the presence of the compound or the respective solvent (control; 0 μM). Shown are arithmetic means±S.E.M. *Significantly different from control ($P<.05$).

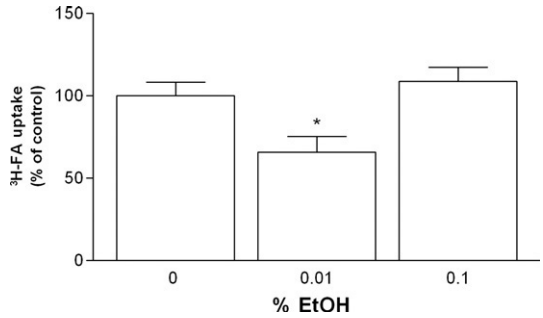

Fig. 2. Effect of the chronic exposure of BeWo cells to ethanol (EtOH; $n=4$–8) 0.01 and 0.1% vol/vol on [^{3}H]FA apical uptake. BeWo cells were cultured for 48 h in the presence of ethanol or standard culture medium (control; 0%). For uptake experiments, cells were incubated at 37°C with 20 nM [^{3}H]FA for 6 min. Shown are arithmetic means±S.E.M. *Significantly different from control ($P<.05$).

3.2. Effect of dietary bioactive compounds on cell viability

In order to assess the cytotoxicity of the compounds that affected the uptake of [^{3}H]FA, we determined BeWo cell viability after acute exposure to epicatechin (100–400 μM), isoxanthohumol (10–400 μM) and theophylline (10–100 μM), and after chronic exposure to ethanol (0.01%), xanthohumol (1–10 μM), isoxanthohumol (10 μM) and quercetin (1–10 μM). We verified that none of the compounds tested (acutely or chronically) decreased cellular viability (results not shown). Interestingly enough, both acute and chronic exposures of BeWo cells to isoxanthohumol (10 μM) slightly increased cell viability [to 115.91±4.71% of control ($n=4$) and 113.55±7.97% of control ($n=4$), respectively], but that increase reached statistical significance in acute treatment only. Chronic exposure of BeWo cells to xanthohumol (10 μM) increased cell viability to 126.65±2.66% of control ($n=4$).

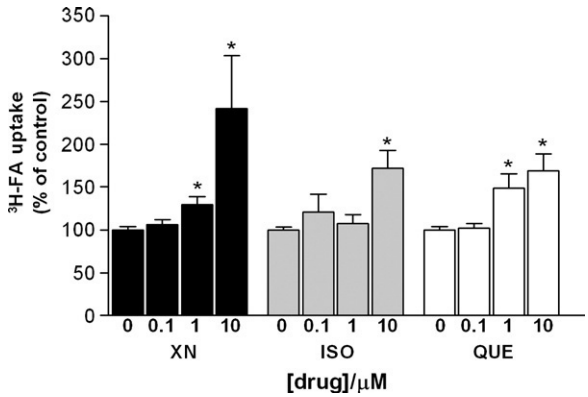

Fig. 3. Effect of the chronic exposure of BeWo cells to different concentrations of the polyphenols xanthohumol (XN; $n=4$–7), isoxanthohumol (ISO; $n=3$–5) and quercetin (QUE; $n=4$–5) on [^{3}H]FA apical uptake. BeWo cells were cultured for 48 h in the presence of different concentrations of the compound or the respective solvent (control; 0 μM). For uptake experiments, cells were incubated at 37°C with 20 nM [^{3}H]FA for 6 min. Shown are arithmetic means±S.E.M. *Significantly different from control ($P<.05$).

3.3. Specificity of the effect of dietary bioactive compounds on [^{3}H]FA uptake

The specificity of the acute and chronic effects of the compounds on [^{3}H]FA uptake by BeWo cells was assessed by testing the effect of these compounds, at the same concentrations, on the uptake of two other nutrients [^{3}H]thiamine and [^{14}C]alanine. As can be observed in Fig. 4A and C, the acute inhibitory effects of epicatechin and theophylline on [^{3}H]FA uptake are specific. However, isoxanthohumol (Fig. 4B) also had an acute inhibitory effect on the uptake of [^{3}H]thiamine and [^{14}C]alanine. Interestingly enough, isoxanthohumol potently and completely inhibited [^{14}C]alanine uptake.

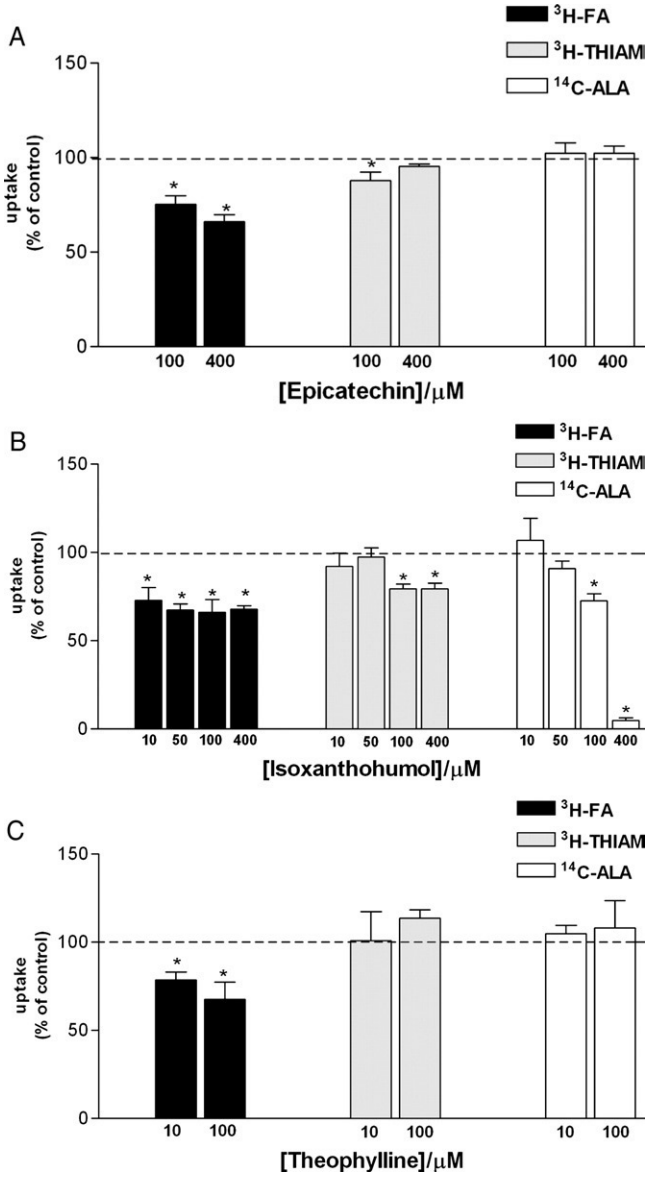

Fig. 4. Specificity of the acute inhibitory effects of (A) epicatechin, (B) isoxanthohumol and (C) theophylline on [^{3}H]FA uptake. BeWo cells were preincubated for 20 min and then incubated with 100 nM [^{3}H]thiamine ([^{3}H]THIAM; n=4–6) for 3 min or with 250 nM [^{14}C]alanine ([^{14}C]ALA; n=4) for 6 min, at 37°C, in the presence of the compound or the respective solvent (control). Shown are arithmetic means±S.E.M. *Significantly different from control (P<.05).

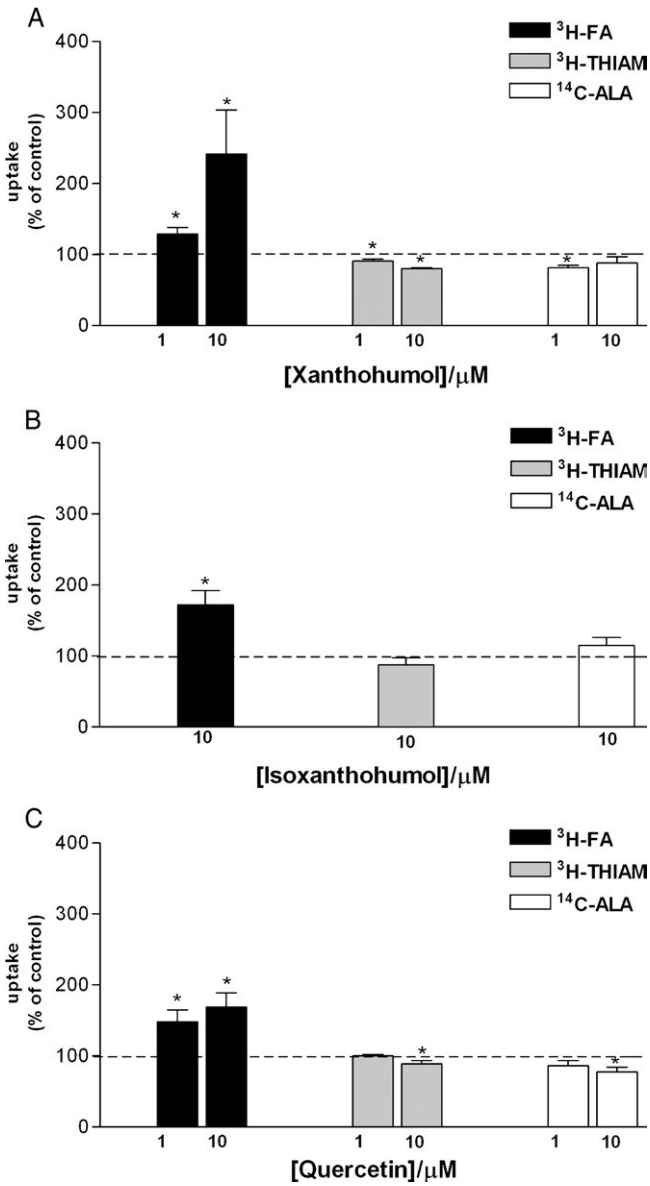

Fig. 5. Specificity of the chronic stimulatory effects of (A) xanthohumol, (B) isoxanthohumol and (C) quercetin on [^{3}H]FA uptake. After the chronic (48 h) exposure of BeWo cells to the compounds, cells were incubated with 100 nM [^{3}H]thiamine ([^{3}H]THIAM; n=4–7) for 3 min or with 250 nM [^{14}C]alanine ([^{3}H]ALA; n=4–9) for 6 min at 37°C. Shown are arithmetic means±S.E.M. *Significantly different from control (P<.05).

Moreover, the chronic stimulatory effects of xanthohumol, isoxanthohumol and quercetin in relation to [^{3}H]FA uptake seem to be also specific, since none of these compounds had a stimulatory effect on the uptake of [^{14}C]alanine or [^{3}H]thiamine (Fig. 5).

3.4. Effect of dietary bioactive compounds on kinetic parameters of [^{3}H]FA uptake

In this set of experiments, we determined the initial rates of [^{3}H]FA uptake (6 min of incubation) at increasing

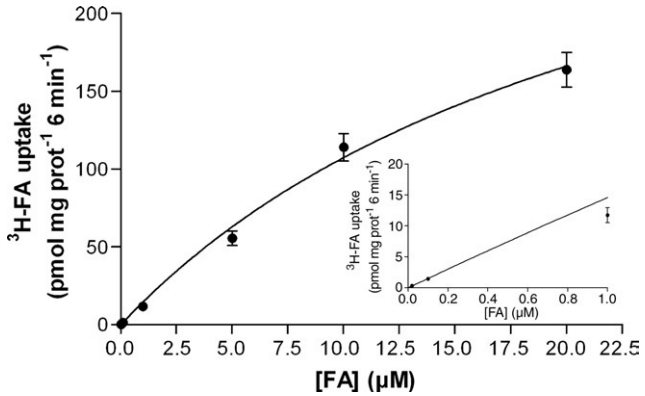

Fig. 6. [^{3}H]FA uptake by BeWo cells as a function of concentration. BeWo cells were incubated at 37°C for 6 min in the presence of increasing concentrations of [^{3}H]FA (0.02–20 μM). Shown are arithmetic means±S.E.M. (n=16).

substrate concentrations (0.02–20 μM) (Fig. 6). The uptake of [^{3}H]FA was found to be saturable with a K_m of 24.2±6.2 μM and a V_{max} of 366.6±58.6 pmol/mg protein/6 min (n=16).

In order to characterize the acute inhibitory effects of epicatechin, isoxanthohumol and theophylline on [^{3}H]FA uptake, we examined the effects of these compounds (at the concentration causing the maximum inhibitory effect on [^{3}H]FA uptake) on the kinetic parameters of [^{3}H]FA uptake by BeWo cells. The results (Table 1) show that all compounds (400 μM epicatechin, 400 μM isoxanthohumol and 100 μM theophylline) induced a significant increase in K_m value (thus reducing the affinity of the transporter to [^{3}H]FA). Moreover, both epicatechin and theophylline caused a significant increase in V_{max} value; isoxanthohumol had no effect on this parameter.

3.5. Effect of dietary bioactive compounds on RFC1 and FRα gene expression in BeWo cells

We first examined the pattern of expression of RFC1 and FRα in BeWo cells under standard culture conditions, using RT-PCR. As shown in Fig. 7, this cell line has been found to express both RFC1 and FRα mRNA.

In order to investigate the nature of the observed chronic effects on [^{3}H]FA uptake, we performed semiquantitative

Table 1
Acute effect of epicatechin (EPI; 400 μM), isoxanthohumol (ISO; 400 μM) and theophylline (THEO; 100 μM) on the kinetic parameters of [^{3}H]FA uptake by BeWo cells

	K_m (μM)	V_{max} (pmol/mg protein/6 min)	n
C	24.2±6.2	366.6±58.6	16
EPI	89.7±33.1*	1219.0±368.4*	12
ISO	63.9±22.6*	577.0±153.3	8
THEO	80.9±11.5*	739.1±87.6*	9

BeWo cells were preincubated for 20 min and then incubated for 6 min at 37°C with increasing concentrations of [^{3}H]FA (0.02–20 μM), in the absence (control; C) or in the presence of epicatechin (400 μM), isoxanthohumol (400 μM) or theophylline (100 μM).
Data are presented as arithmetic means±S.E.M.
* Significantly different from control (P<.05).

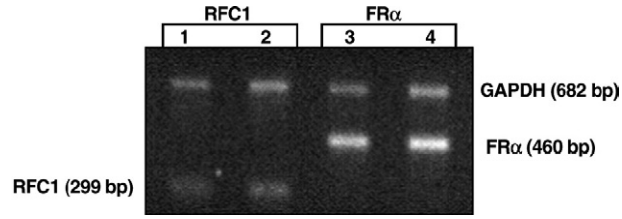

Fig. 7. Detection by RT-PCR of RFC1 and FRα mRNA in total RNA from BeWo cells. RT-PCR analysis was performed with specific primers for RFC1 (Lanes 1 and 2) and FRα (Lanes 3 and 4). As control for the intactness of mRNA, GAPDH mRNA was detected with specific primers. PCR products were separated by agarose gel electrophoresis, followed by ethidium bromide staining.

RT-PCR with total RNA from BeWo cells chronically treated with the compounds that had a significant effect on the vitamin transport.

Xanthohumol, isoxanthohumol and quercetin (all at 10 μM) did not affect RFC1 or FRα gene expression. The observed levels of RFC1 expression were 96.61±4.88%, 115.43±8.29% and 85.77±5.79% of controls, and observed levels of FRα expression were 89.16±14.95%, 87.66±

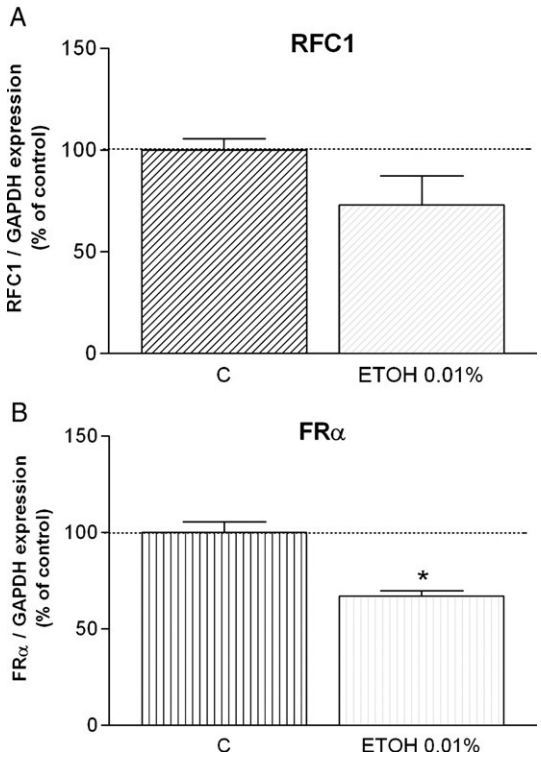

Fig. 8. Analysis of mRNA levels for (A) RFC1 and (B) FRα in BeWo cells chronically exposed to ethanol 0.01% (EtOH; n=4). BeWo cells were treated with 0.01% ethanol or standard culture medium (control; C) for 48 h at 37°C. Total RNA was then isolated from these cells and used for semiquantitative RT-PCR. Specific primers for RFC1 and FRα were used. As control for the intactness of mRNA, GAPDH mRNA was detected with specific primers. PCR products were separated by agarose gel electrophoresis, followed by ethidium bromide staining. Relative band densities RFC1/GAPDH (A) and Frα/GAPDH (B) were calculated. RFC1/GAPDH and FRα/GAPDH ratios in control cells were taken as 100%. Shown are arithmetic means±S.E.M. of two determinations from two independent experiments. *Significantly different from control (P<.005).

15.60% and 89.13±3.37% of controls, for chronic exposure to xanthohumol, isoxanthohumol and quercetin (all at 10 μM), respectively. However, chronic exposure of BeWo cells to ethanol (0.01%) caused a significant reduction in FRα (but not RFC1) mRNA levels (Fig. 8). This reduction was quantitatively very similar to the reduction of [^{3}H]FA uptake caused by the same concentration of ethanol (Fig. 2) (67.20±2.62% and 65.82±9.40% of controls for FRα expression and [^{3}H]FA uptake, respectively; n=4).

4. Discussion

The aim of this work was to investigate the effect of some bioactive substances present in alcoholic or nonalcoholic drinks on FA placental absorption. For this purpose, we determined both the acute and the chronic effects of these compounds on [^{3}H]FA uptake by BeWo cells. The BeWo cell line derives from human gestational choriocarcinoma and is a well-known cellular model of human syncytiotrophoblast [33,34], having been much used to investigate placental trophoblast transport function for a number of compounds. BeWo cells, besides exhibiting morphological properties, producing biochemical marker enzymes and secreting hormones characteristic of normal trophoblasts, rapidly form a confluent polarized monolayer, which is particularly attractive for studies on transplacental barrier [33]. Indeed, they have been shown to exhibit polarized nutrient uptake systems [35,36] and polarized transcellular transport of transferrin [37] and serotonin [38].

In a recent work, we have characterized [^{3}H]FA uptake in BeWo cells [24]. Our results suggested that, at physiological pH, [^{3}H]FA apical uptake by these cells involves both RFC1 and FRα. This conclusion was based on the following observations: the 6-min uptake of 0.02 μM [^{3}H]FA by BeWo cells at pH 7.5 was: (a) inhibited by the FA analogue methotrexate; (b) *cis*-inhibited and *trans*-inhibited by a high load of unlabeled FA; (c) inhibited by the anion transport inhibitor 4-acetamido-4′ -isothiocyanato-2,2′ -stilbenedisulfonic acid; (d) inhibited by an anti-RFC antibody; and (e) inhibited by monensin, an inhibitor of receptor-mediated endocytosis. Moreover, in the present work, a saturable carrier-mediated process was defined, with a K_m of 24.2± 6.2 μM and a V_{max} of 366.6±58.6 pmol/mg protein/6 min.

In the present work, we report for the first time that BeWo cells express both RFC1 and FRα mRNA. So, we also determined by RT-PCR the chronic effects of bioactive substances on the expression of both RFC1 and FRα mRNA.

First, we investigated the acute and chronic effects of cell exposure to ethanol because this agent is recognized as a potent teratogen in humans [39]. Indeed, prenatal alcohol exposure can give rise to alcohol-related birth defects such as spontaneous abortion, decreased immune function, attention problems, hearing impairment [40] and, as mentioned above, FASD [41]. Among children with FASD, a small population presents a specific set of anomalies (specific facial abnormalities, intrauterine growth retardation and significant impairments in neurodevelopment) [39,42–44], known as fetal alcohol syndrome (FAS). FAS is the major known cause of mental retardation in the western world [44] and occurs in 1.9 per 1000 live births [42].

Although it is known that ethanol freely crosses the placenta, being both placentotoxic and fetotoxic [45,46], the exact mechanisms underlying its toxicity are not completely understood. It comes out from the literature that FASD is multifactorial, arising from several possible mechanisms of alcohol action: (a) disruption of midline serotonergic neuronal development [41]; (b) alterations in cell adhesion molecules [41]; (c) fetal hypoxia and free radical formation [40,41]; (d) interference with growth factor signaling [41]; and (e) disruption of cellular growth and differentiation [41,45]. In addition, there is some evidence that alcohol toxicity during pregnancy is associated with altered placental transport function [47–51]. FA is essential for normal fetal development, and disturbances in FA homeostasis have been controversially associated with ethanol placentotoxicity and fetotoxicity.

In this work, we verified that, acutely, ethanol (0.1–5% vol/vol) exposure did not affect [^{3}H]FA uptake by BeWo cells. In contrast, chronic exposure of BeWo cells to 0.01% vol/vol (but not 0.1% vol/vol) ethanol significantly inhibited [^{3}H]FA uptake (to about 65% of control). Presently, we have no explanation for the observation of an inhibitory effect of ethanol at the lowest concentration only. However, it is possible that the highest concentration of ethanol affects membrane permeability properties and prevents us from seeing its effect on transporter-mediated process(es).

Importantly, inhibition by 0.01% ethanol was accompanied by a quantitatively similar reduction in FRα (but not RFC1) mRNA levels, thus suggesting that reduction of [^{3}H]FA uptake may be due, at least in part, to a reduction in FRα gene expression. This observation reinforces our previous results suggesting that FRα is involved in apical [^{3}H]FA uptake in this cell line [24]. Moreover, these results are in strong agreement with previous observations by other groups suggesting, on one hand, that acute exposure to ethanol has no effect on maternal-to-fetal transfer of 5-methyltetrahydrofolate by the perfused human placental cotyledon [47] and, on the other hand, that chronic exposure of dams to ethanol causes a significant reduction in FR activity [51]. Despite this apparent dicotomy, ethanol toxicity has been suggested for a long time to be associated with FA deficiency [6] arising both from reduced FA transport at several tissue levels (namely, reduced intestinal uptake [52–54], reduced hepatic uptake [55] and reduced renal reabsorption [56–59]) and from malnutrition [60]. Our work, showing that chronic ethanol exposure decreases both placental FA uptake and FRα mRNA levels, suggests that a negative interaction between chronic ethanol consumption and FA transport does also exist at the placental level.

To further investigate the nutritional modulation of FA placental uptake, we also determined the effect of acute and chronic exposure of BeWo cells to different polyphenols

(catechin, chrysin, epicatechin, epigallocatechin-3-gallate, isoxanthohumol, myricetin, quercetin, resveratrol, rutin and xanthohumol) and methylxanthines (caffeine and theophylline) present in alcoholic and nonalcoholic drinks on [^{3}H]FA uptake by BeWo cells. The current increasing interest in studying polyphenols arises from their antioxidant properties and their potential role in the prevention of cancer and cardiovascular, neurodegenerative and inflammatory diseases [61–65]. It is known that polyphenols, as well as methylxanthines, freely cross the placenta [66,67]; therefore, the effects of these bioactive compounds on the placenta and on the fetus should be investigated.

Firstly, we observed that, acutely, epicatechin (100 and 400 μM), isoxanthohumol (10–400 μM) and theophylline (10 and 100 μM) inhibited [^{3}H]FA uptake by BeWo cells. After the analysis of the effect of each compound on the kinetic parameters of [^{3}H]FA uptake by these cells, we conclude that isoxanthohumol seems to be a competitive inhibitor, reducing the transporter's affinity for the substrate (it induces an increase in K_m and does not change V_{max}). On the other hand, both epicatechin and theophylline show an atypical behavior because they cause an increase in both K_m and V_{max}. It can be hypothesized that these compounds bind to an allosteric site of the transporter and induce an alteration in the conformation of the active site, thus reducing the affinity for the substrate (increasing K_m) and that, simultaneously, this binding increases the transporter's capacity (increasing V_{max}) for high concentrations of the substrate. Nevertheless, the effect of these two compounds on the kinetic parameters of [^{3}H]FA uptake is in good agreement with their effect on [^{3}H]FA uptake, which was measured with an [^{3}H]FA concentration (20 nM) well below the K_m value of the transport.

Secondly, on chronic exposure, xanthohumol, quercetin (1 and 10 μM) and isoxanthohumol (10 μM) caused an increase in [^{3}H]FA uptake by BeWo cells. The increase in [^{3}H]FA uptake caused by chronic exposure of BeWo cells to the referred polyphenols was not accompanied by a change in RFC1 or FRα mRNA levels and, thus, they do not seem to result from the modulation of the expression levels of these transporters. These chronic effects may otherwise be a result of a direct interaction of the polyphenols with the transporter(s), with consequent change in the activity of the latter.

In summary, our results suggest, on one hand, a detrimental effect of acute epicatechin, isoxanthohumol and theophylline on placental FA absorption and, on the other hand, a benefic effect of chronic xanthohumol, isoxanthohumol and quercetin on FA absorption at the placental level. Moreover, our results also show that both acute and chronic effects of the compounds on [^{3}H]FA uptake do not result from cytotoxic effects on cells and are specific.

Beverages such as tea and red wine constitute important sources of polyphenols [65]. Quercetin is one of the most ubiquitous polyphenol and is present in wine, beer and tea [65,68,69]. Catechin, epicatechin and epigallocatechin-3-gallate are found in black and green teas [65,68]. Xanthohumol, a prenylated chalcone that occurs only in hop plants, and its related prenylflavonoid isoxanthohumol are found in beer [70]. Caffeine and theophylline are well-known methylxanthines present in drinks such as coffee and tea.

In conclusion, this work shows that [^{3}H]FA apical uptake by BeWo cells is modulated by several dietary bioactive compounds. Acutely, uptake of this vitamin by BeWo cells was significantly reduced by exposure to epicatechin (100 and 400 μM), isoxanthohumol (10–400 μM) and theophylline (10 and 100 μM); isoxanthohumol seemed to act as a competitive inhibitor, whereas epicatechin and theophylline caused an increase in both K_m and V_{max}. Moreover, [^{3}H]FA apical uptake by BeWo cells was significantly increased by chronic exposure to xanthohumol, quercetin (1 and 10 μM) and isoxanthohumol (10 μM). This increase does not seem to result from a change in RFC1 or FRα gene expression. Moreover, we have also demonstrated that [^{3}H]FA uptake is significantly reduced by the chronic exposure of BeWo cells to 0.01% ethanol, with this reduction being, at least in part, due to a reduction in FRα expression. These results suggest that one possible mechanism underlying ethanol toxicity in pregnancy, hence the development of FASD, may indeed be the inhibition of placental FRα gene expression and the consequent insufficient FA supply to the placenta and the fetus.

Finally, it is worth noticing that acute and chronic treatments with all the dietary bioactive compounds did not produce parallel results and, therefore, care should be taken when speculating about chronic effects from acute effects and vice versa.

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