Quercetin Metabolism and Transport Dynamics in the Presence of Benzo(a)pyrene: An In Vitro Investigation

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ABSTRACT

Quercetin is a flavonoid able to modify the metabolism of carcinogen benzo(a)pyrene in human intestinal cells but it is not known if its metabolism is affected by the presence of B(a)P. In this study the phase-2 metabolism and transport rate of quercetin has been studied using CaCo-2/TC7 cell line, model of human enterocytes, co-treated with B(a)P. Quercetin and its main metabolites, sulfated and glucuronidated, were quantified at different times (from T0 to T 24h), using HPLC with diode array and MS detection. This study showed that quercetin metabolism in CaCo-2/TC7 cells co-treated with B(a)P presents some differences respect to control in the formation of sulphates coniugates, more evident in the last time of analysis. The transport study, instead, confirmed a stronger influence of BaP with an increased rate of glucuronidation and sulfation of quercetin in both cellular side. The effects of B(a)P observed on phase-2 metabolism and transport rate of quercetin, by *in vitro* model of human enterocytes, put in evidence its possible influence, consequently, also on biological activities of this flavonoid.

Keywords: Benzo(a)pyrene, quercetin, CaCo-2 cell line, metabolism, transport, HPLC-MS.

INTRODUCTION

Diet represents a significant source of exposure to contaminants and food–borne carcinogens as benzo(a)pyrene but, at the same time, a font of assumption of phytochemicals, as quercetin, with protective effects for human health. Many studies showed a correlation between high dietary intake of cooked meat and an increased risk of cancer of colon, pancreas, liver, prostate and breast[1] due to the presence of food-borne cancerogens and mutagens.

Benzo(a)pyrene (BaP) is a marker of cancerogenicity of polycyclic aromatic hydrocarbons (PAHs), produced during cooking processes (drying, boiling, cooking, grilling, roasting, toasting, smoking)[2-3] for combustion of organic matter. Due to their physical and chemical properties, BaP has an high solubility in lipids, can migrate through the food chain into hydrophobic compartments and be retained by food rich in fats[4-6]. At the same time, it could be present in food as consequence of environmental pollution[7], in fact substantial amounts of this toxic compound were found also in vegetable foods (bread, cereals, grains, fruits, etc.)[8-9].

Epidemiological studies, instead, have shown that diets rich in fruit and vegetables are associated with a lower risk of developing food-related malignancies[10-11], for the presence of phytochemical compounds (polyphenols, flavonoids, antocyanidines, isotyocyanates ect.), considered natural anti-carcinogens[12-14]. Among these phytochemicals, quercetin is a major flavonoid present in various fruits (as blackberry, mulberry, apple, etc.) and vegetables (as onion, broccoli, etc.), with a wide range of *in vitro* biological activities[15], such as antinflammatory, antioxidant anticancer, antidiabetic, etc.[16-18].

Therefore, at present, there is an increasing interest to assess the fate of food contaminants and phytochemical compounds after food ingestion and their possible interactions in the gastrointestinal tract. Particularly, the CaCo-2 cell line, is widely used as a model to study human intestinal transport and metabolism, biotransformation and permeability[19]. The CaCo-2 cell line, established from a human colon adenocarcinoma, has retained the ability to differentiate into polarized epithelial monolayers, shows numerous biochemical and morphological characteristics of enterocytes (e.g., formation of microvilli, tight junctions and desmosomes, expression of brush-border enzymes such as sucraseisomaltase)[20] and expresses various phase-1 and phase-2 enzymes. In particular, the CaCo-2 sub-clone TC7, generated by passaging CaCo-2 cells 198 times[21], is characterized by a selection of faster growing cells, shorter population doubling time, higher cell density and full differentiation after a shorter period of time, in respect to the parent population.

The absorption and metabolism of many bioactive compounds, like quercetin, has been studied in CaCo-2 cell line[22-23]. Quercetin aglycone is initially absorbed in gastrointestinal tract by passive diffusion[24-25], then hydrolyzed and further conjugated within the enterocytes

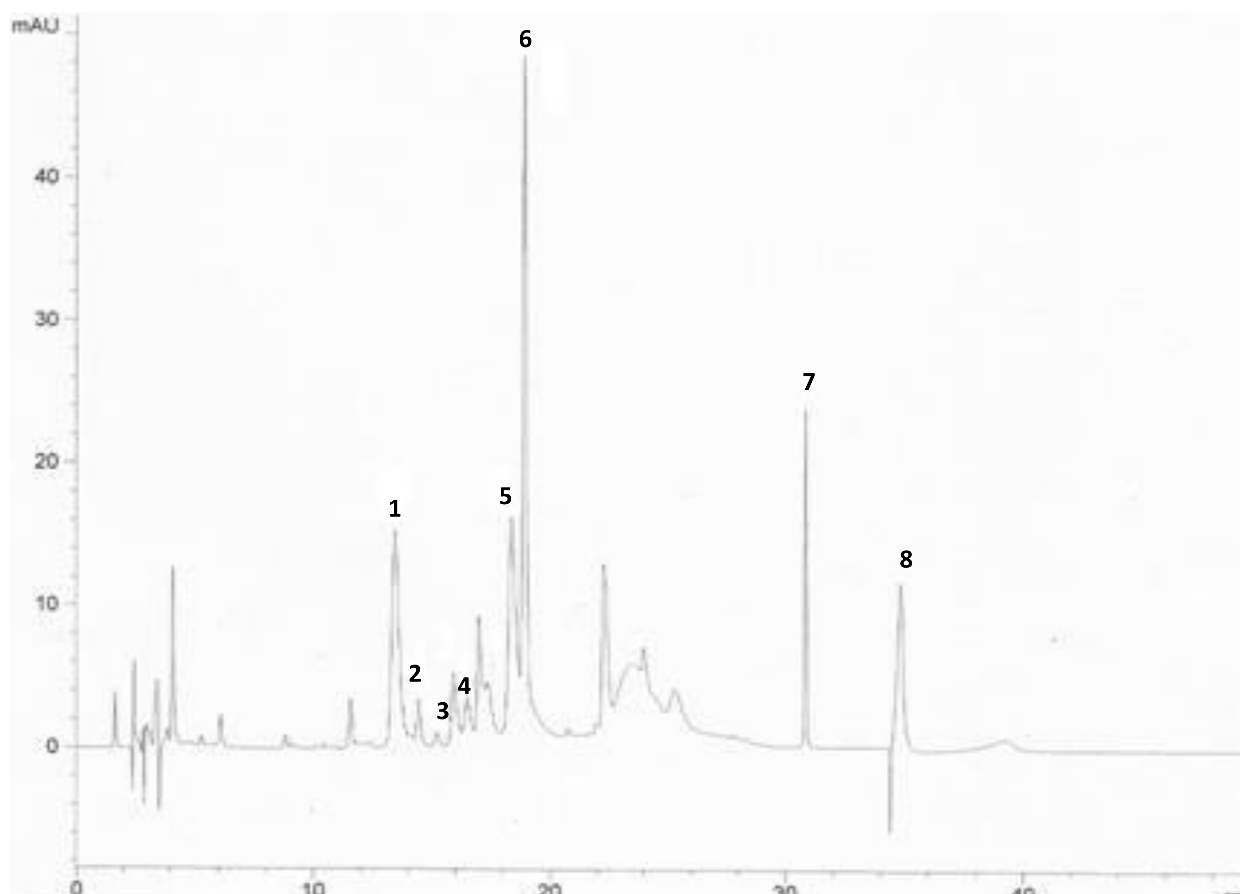


Figure 1: Chromatographic profile of quercetin and its main metabolites in co-treatment with B(a)P.

1. quercetin-7-glucuronide (Q-7-GlcA); 2. quercetin-3'-glucuronide (Q-3-GlcA); 3. 3'methylquercetin-3-glucuronide (IR-3-GlcA); 4. quercetin-4'-glucuronide (Q-4'-GlCA); 5. quercetin-3-glucuronide (Q-3-GlcA); 6. quercetin (Q); 7. quercetin-3-sulphate (Q-3-S); 8. benzo(a)pyrene B(a)P

by enzymes, to form a number of phase-2 conjugates, most notably sulphates and glucuronides[26-28]. Finally quercetin conjugates are excreted by the liver, reducing their bioavailability.

Similarly, BaP transport[29-30], uptake and metabolism[31] have been investigated in CaCo-2 cell line[32-33]. Some authors have studied the effects of polyphenols and flavonoids[34-35], like ellagic acid[36] and chrysin[37], on metabolic activation[13], cellular excretion[38] and transport[39] of benzo(a)pyrene but no data are reported on influence of this carcinogen on metabolic behaviour of phytochemical compounds as quercetin. The aim of this study, therefore, is to evaluate the effects of BaP on phase 2-metabolism of flavonoid quercetin and its cellular transport rate in CaCo-2 cell line, co-exposed to this toxic compound.

MATERIALS and METHODS

CaCo-2/TC7 cell culture

The clonal line CaCo-2/TC7, derived from parental CaCo-2 cells, obtained from Dr Monique Rousset (INSERM, Paris, France), were cultured between passages 32 and 51. Cells were grown in Dulbecco Modified Eagle's Medium (DMEM) with 1% nonessential amino acids, 1% L-glutamine, 100 IU/ML penicillin and 100 µg/ml streptomycin, supplemented with 20% (v/v) foetal calf serum (FCS). At first, cells were sub-cultured 5-6 days post seeding. Cell were seeded at 2-4 × 10^4 cells in 75 cm^2 flask and sub-cultured 5-6 days post seeding when the cells reached 80% confluency observed under a light microscope. Finally, cells were seeded at 2-4 × 10^4 cells per cm^2 on 10 cm dishes (growing area 75 cm^2) and allowed to grow until 21 days post confluent, changing media 3 daily. *Chemicals*

Quercetin standard solution was purchased from Extrasynthese, 69727 Genay Cedex, France. Quercetin-3glucuronide (Q3glA), quercetin-3'-glucuronide (Q3'glA), quercetin-4-glucuronide (Q4glA), quercetin-7glucuronide (Q7glA), and 3-methylquercetin-3glucuronide (isorhamnetin-3-glucuronide, IR3glA were chemically synthesized at the Institute of Food Research,

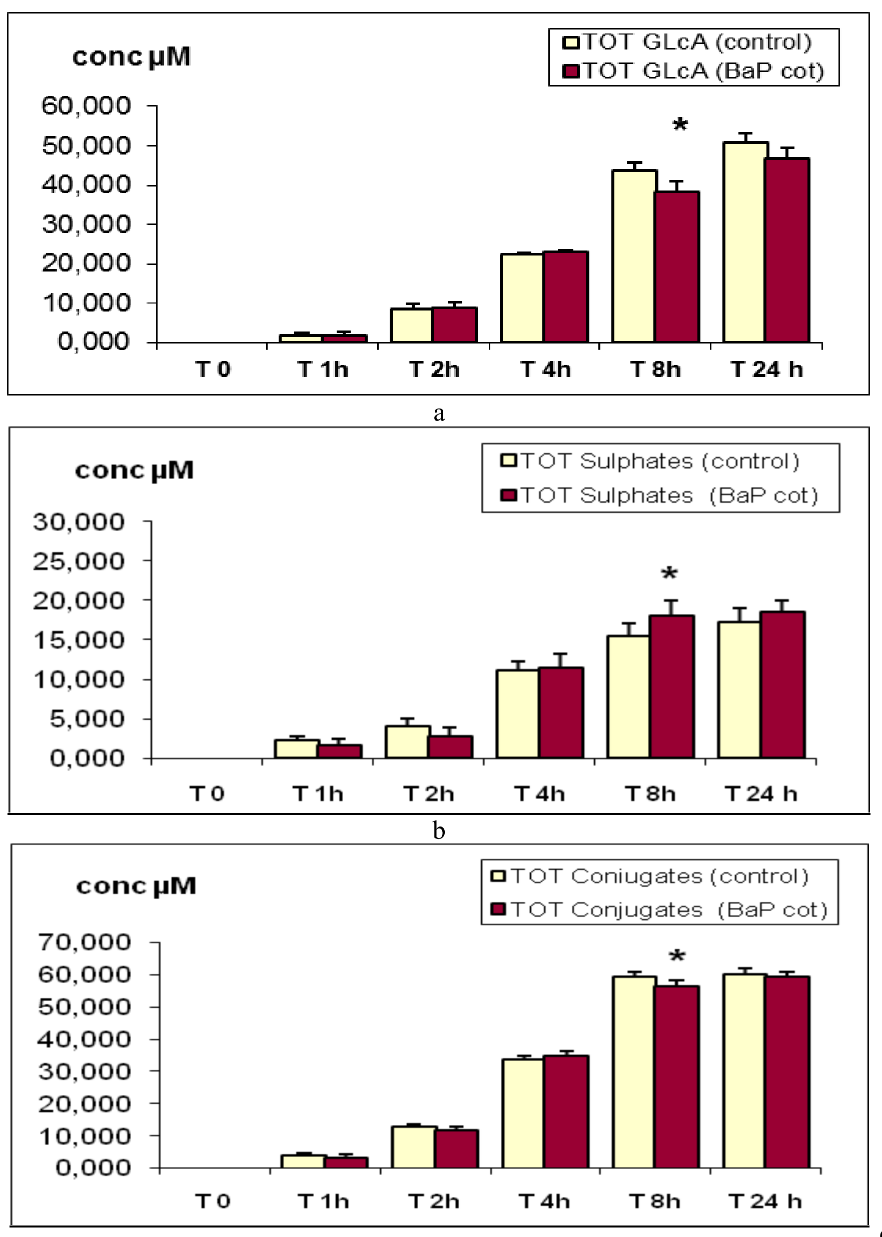


Figure 2: Quercetin's metabolites rate, in presence of benzo(a)pyrene (BaP cot), expressed as *a)* total glucuronides (*TOT GlcA*), *b)* total sulphates (*TOT Sulphates*) and *c)* total conjugates (*TOT Conjugates*). **P<0.05*

Norwich[39] while quercetin 7-sulphate was provided by Dr Denis Barron, Nestle ResearchCentre, Lausanne, Switzerland. Purity of compounds was checked by HPLC with UV detection and was 98.5% for all. Benzo(a)pyrene standard solutions, all solvents and other reagents of analytical grade were obtained from Sigma– Aldrich, Poole, UK.

Metabolism studies

Cells were treated, in triplicate, with solution of quercetin (60µM) as control, co-treated with quercetin (60µM) and benzo(a)pyrene (10 µM) and DMSO was used as analytical blank. After incubation, aliquots of extracellular culture samples at different times (T 0, 30 min, 1h, 2h, 4h, 8h and 24h) were collected and treated with 20µl of Acetonitrile and Formic Acid, stored at 20°C, centrifuged and transferred in vials for chromatographic analysis[22].

A stability study of quercetin in presence of BaP was also carried out in fresh and spent media (after 2h of incubation); media samples, collected at different times of incubation (T 0, 2h, 4h and 8h), were treated in the same conditions previously described.

Preparation of CaCo-2/TC7 monolayers for transport studies

A CaCo-2/TC7 monolayer was prepared by seeding cells (from 75 cm^2 flasks) at 2-4 x 10^4 cells per cm^2 on 12 – well of 0.65 µm (0.33 cm^2) Transwell polycarbonate filters (Corning Costar Corporation, Sigma- Aldrich, UK) and grown to confluence for 21 days. The media was changed every 2 days and cells were allowed to grow until 21 days post confluent. The evaluation of monolayer integrity of the cells in the Transwells was determined by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS volt ohmmeter (Millipore corporation, Billerica, Massachusetts, USA)[40]. TEER measurements of 6 and 12 well Transwells monolayers were routinely made prior to changing the cell culture media; additionally TEER measurements were taken also before and at the end of experiments to ensure adequate monolayer integrity.

Table 1: Metabolic behaviour of quercetin (60 µM) in cotreatment with benzo(a)pyrene (10 µM).

	Q-7-GlcA	Q -3'-GlcA	IR Q-3-GlcA	Q-4'-GlcA	Q-3 -GlcA	QUERCETIN	Q-3'- S
T 0	conc µM						
QUE (control)	n.d.	n.d.	n.d.	n.d.	n.d.	60.635 ± 11.72	n.d.
QUER (BaP ct)	n.d.	n.d.	n.d.	n.d.	n.d.	60.184 ± 10.84	n.d.
T 1h	conc µM						
QUE (control)	0.806 ±0.45	n.d.	n.d.	n.d.	0.981 ± 0.040	45.58 ± 7.24	2.259 ± 0.831
QUER (BaP ct)	0.939 ±0.26	n.d.	n.d.	n.d.	0.821 ± 0.088	40.894 ± 8.67	1.639 ± 0.584
T 2h	conc µM						
QUE (control)	4.413 ± 0.50	0.415 ± 0.079	0.253 ± 0.027	0.431 ±0.017	3.221 ± 0.583	33.971 ± 3.464	4.063 ± 0.556
QUER (BaP ct)	5.297 ± 1.211	0.670 ±0.194	0.202 ± 0.016	0.280 ± 0.045	2.399 ± 0.143	30.711 ±6.424	2.857 ± 0.853**
T 4h	conc µM						
QUE (control)	10.562± 1.51	1.107 ± 0.24	0.591 ± 0.036	1.316 ± 0.322	8.914 ± 0.997	15.851 ± 2.682	11.239 ±0.765
QUER (BaP ct)	12.084 ±2.185*	1.068 ± 0.113	0.542 ± 0.045	1.187 ± 0.074	8.283 ±1.051	9.062 ±1.091**	11.520 ±2.722
T 8h	conc µM						
QUE (control)	16.558 ±4.438	3.205 ± 0.581	1.499 ±0.349	3.439 ±0.508	19.001 ±4.793	0.217 ±0.029	15.528 ±1.318
QUER (BaP ct)	15.189 ±3.919	2.490 ±0.373*	1.738 ±0.137	3.635 ±0.757	15.309 ±3.139**	0.186 ±0.041*	18.116 ±1.645**
T 24h	conc µM						
QUE (control)	17.945±0.991	3.378 ±0.660	2.112 ±0.437	4.413 ±0.816	21.089 ±1.244	n.d	17.225±0.686
QUER (BaP ct)	18.749 ±2.055	2.719 ±0.472	2.107 ±0.088	4.412 ±0.808	19.938 ±3.260*	n.d	18.543±2.693

*$P<0.05$**$P<0.01$*

Transport studies

From apical and basolateral compartments of CaCo2/TC7 monolayer, the media was removed by aspiration and 0.2 ml and 1 ml of samples were added in apical and basolateral side, respectively. To study quercetin transport in co-treatment with BaP, in Transwell system of 0.65 µm (0.33 cm^2), cells were treated, in both apical and basolateral side, in triplicate, with solution of quercetin (60µM) used as control, co-treated with quercetin (60µM) and benzo(a)pyrene (10 µM), while DMSO was used like analytical blank. Aliquots collected at different time after incubation (T 0, 15min, 1h, 2h, 4h, 8h and 24h) were treated with 20µl of Acetonitrile and Formic Acid and analyzed as previously described. *Determination of apical to basolateral ratio from transport experiments*

To calculate the amount (µM) of flavonoids (aglycone and all metabolites) in the apical and basolateral side, the peak

area from the HPLC chromatograms was considered. The apical to basolateral ratio is expressed by the equation below: ratio = apical efflux basolateral efflux

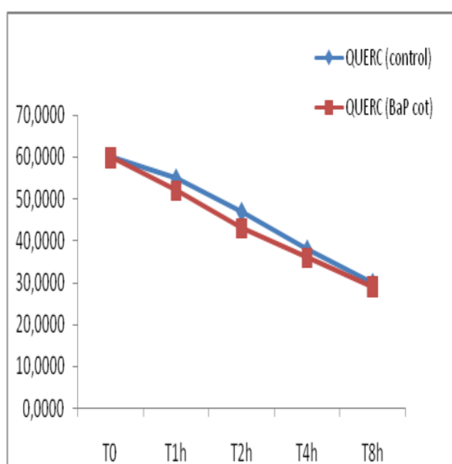
This ratio is a measure of the favoured direction of efflux, where value of over 1.0 indicating an apically favoured efflux, values below 1.0 symbolising a basolaterally favoured efflux, and values of 1.0 symbolising an equal distribution of conjugate efflux.

HPLC analysis

The study of quercetin metabolism was realized in an Agilent 1100 HPLC (Agilent, Stockport, UK) comprising of two pump units, auto-sampler, mixer, and a diodearray detector at λ_{max} 270 and 370 nm, using a Phenomonex Luna C18 column (250 mm x 4.6 mm i.d.) 5 µm equipped with guard column. Samples were eluted using like mobile phase MilliQ water (A) and Acetonitrile (B) with 0.1% TFA, at this elution gradient: 17% (B) from 0 to 2 min ; 25% B from 2 to 7 min; 35% B from 7 to 15 min; 50% from 15 to 20 ; 100% B from 20 to 25 min; 100% B until 30 min; 17% B from 30 to 35 min, followed until 50 min. Analysis was conducted at temperature of 20 C°, at flow of 1 ml/min and injection volume was 50 µl.

To validate the chromatographic method used, all specific parameters were assessed. The limit of detection (LOD) for quercetin and its metabolites was determined as 0.007 µM. To check the linearity, standard solution of quercetin and its metabolites at different concentrations (40, 20, 10 µg/ml) were analysed. Calibration curves for each compound were constructed using the linear least-squares regression procedure (n=4) of peak area versus standard concentration in µg/ml (R^2=0.999). The accuracy and repeatability of the method was assessed by performing a spike-and-recovery test. Recovery was measured using fortified samples (n = 3 replicates) each at three levels of concentration, corresponding to 85 %, 100% and 110%, respectively, for each analyte. Spike recoveries were repeated three times for each concentrations and the results were expressed as average percentage of recovery. The specificity was confirmed by analysis of blank samples.

To confirm quercetin metabolites detection by HPLC, further analysis were performed using LC with mass spectrometry in positive mode (Micromass Quattro II; Manchester, UK)[22]. Chromatographic runs were carried out under the same conditions previously described. The limit of detection in culture media samples was determined as 0.004 µM.



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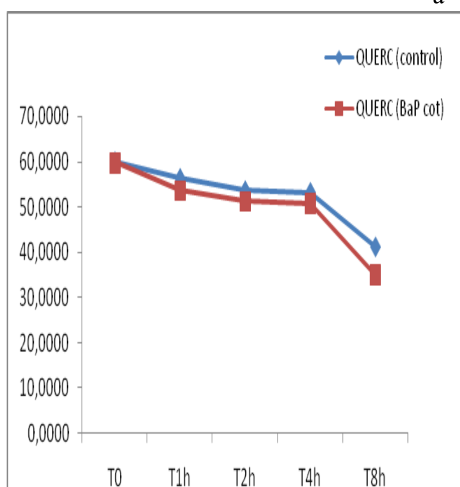

b

Figure 3: Quercetin stability test in fresh (a) and spent (b) media in presence of B(a)P.

Statistical analysis
The results were reported as mean value ± S.D. from a minimum of three replicate measurements. When total conjugates, glucuronates (GlcA) and sulphats are reported, the data were obtained by summing all the values for the individual conjugates but did not include the free quercetin. Data were submitted to analysis of variance (ANOVA) and paired sample *t*-test to determine the significance of differences between groups. P values <0.01 and <0.05 were accepted as statistically significant.

RESULTS

Metabolism studies
The metabolic profile of quercetin and its main metabolites (quercetin-7- glucuronide, quercetin 3'- glucuronide, IR-quercetin 3- glucuronide, quercetin 3- glucuronide, quercetin 4'- glucuronide, quercetin 3- sulphate), identified and quantified in co-treatment with Benzo(a)pyrene through the chromatographic analysis, is reported in Figure 1.
The concentrations of quercetin and its metabolites in cells co-treated with BaP at different times, in comparison to the control, are reported in Table 1. In

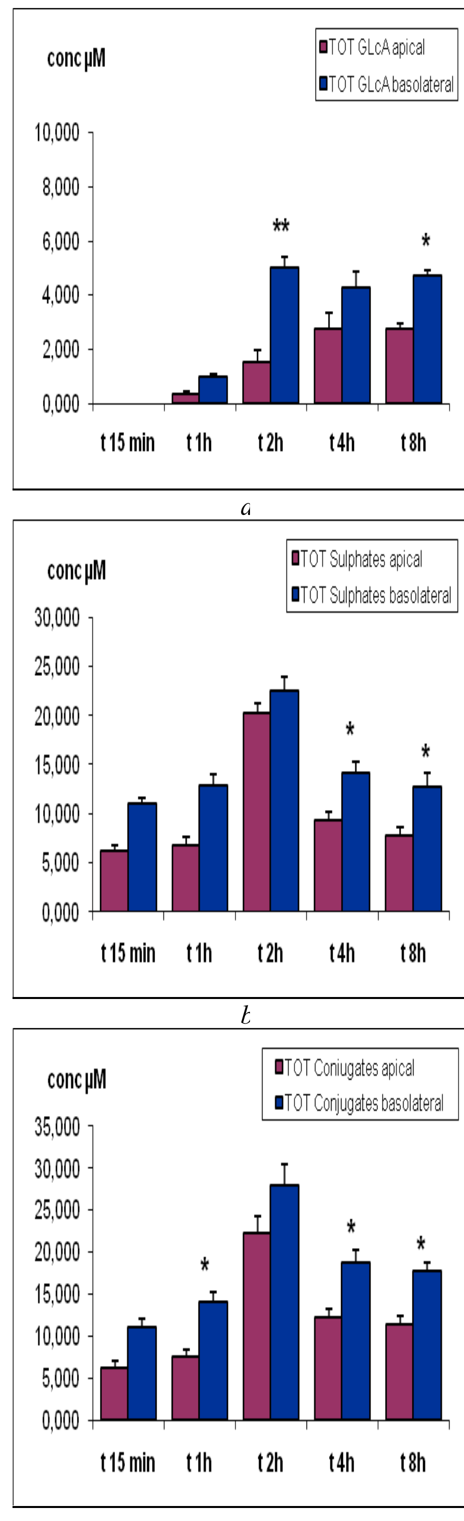

Figure 4: Comparison between quercetin's transport rate, in apica
benzo(a)pyrene, expressed as total GlcA *(a)*, total Sulphate *(b)*, to

particular, from these data it's possible to observe that quercetin decreases significantly at 4h (P<0.01), respect to control, until to be absent at 24h (considered the last step of metabolic process). All main metabolites of quercetin are present after 2h; their concentrations present same differences (P<0.05 and P<0.01) in presence of BaP during the intermediate time of analysis (4h and 8h), reacting at

24h values lower than control, except for Q-7GlcA and Q-3'-S. These results were expressed also in Figure 2 as sum of glucuronates metabolites (Tot GlcA), sulphate metabolites (Tot Sulphates) and total conjugates (Tot conjugates), except free quercetin. In presence of BaP the production of glucuronates metabolites is similar to control until 4h but decreases significantly from 8 h to 24h, respect to control (Fig 2a), instead, the rate of sulphates metabolites is higher than control from 8h to 24h (Fig 2b). Considering the total production of phase 2metabolites (Fig 2c), it's possible to observe that in presence of BaP the total amount of conjugates is similar to control at 4h but decreases from 8 h until to the last metabolic step at 24h.

In Figure 3 is reported the study of quercetin stability in presence of BaP, in fresh and spent media (after 2h of incubation). The results obtained showed a similar trend in both experimental conditions, with a gradual loss of quercetin from 1h to 8h in fresh media (Fig 3a), more evident at 4h and 8h in "spent" media (Fig. 3b), possible due to the slow auto-oxidation of quercetin in media.

Table 2: Transport rate (conc µM) of quercetin in apical side, in co-treatment with benzo(a)pyrene.

T 0	QUERCETIN *conc µM*	TOT GlcA	TOT Sulphate	TOT Conjugates
QUE (control)	59.94± 9.324	-	-	-
QUER (BaP ct)	59.79±8.187	-	-	-
T 30 min	*conc µM*			
QUE (control)	53.36±7.328	0.132±0.065	4.362±0.837	4.494±0921
QUER (BaP ct)	52.499±5.492	-	6.242±1.328	6.242±1.538
T 1 h	*conc µM*			
QUE (control)	49.06±5.033	0.788±0.033	6.839±1.243	8.331±1.738
QUER (BaP ct)	49.064±7.257	0.355±0.048	6.839±1.025	7.529±0.937
T 2h	*conc µM*			
QUE (control)	22.67±3.176	1.823±0.656	5.332±0.963	7.727±1.533
QUER (BaP ct)	17.473±4.226*	1.528±0.092	20.215±1.985**	22.242±4.281**
T 4h	*conc µM*			
QUE (control)	4.477±0.983	3.044±0.927	7.009±1.052	10.481±1.772
QUER (BaP ct)	7.174±2.117	2.758±0.671	9.307±1.143	12.281±2.004
T 8h	*conc µM*			
QUE (control)	0.425±0.084	2.328±0.821	6.631±0.972	10.349±1.637
QUER (BaP ct)	0.184±0.028	2.735±0.573	7.800±1.297	11.376±2.148

*$P<0.05$ **$P<0.01$

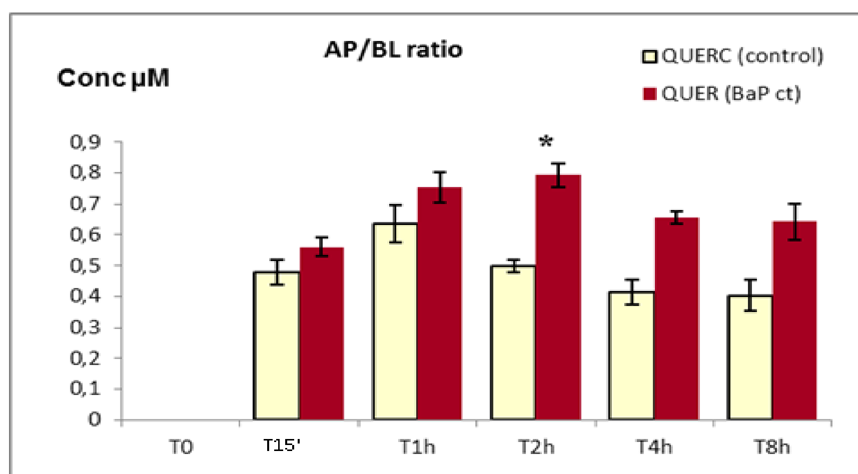

Figure 5: Quercetin transport rate, in co-treatment with benzo(a)pyrene, expressed as Apical/Basolateral ratio of total conjugates. *$P<0.05$

Transport studies

Data related to the transport of quercetin and its metabolites in apical and basolateral side of cells, in presence of BaP, are reported in Table 2 and 3, respectively. In apical side, quercetin concentrations in

cells co-treated with BaP decreased respect to control, except to 4h; the total glucuronates content is less than control, but the sulphates are increased for all time of analysis with a maximum value at t 2h, with statistically significant differences (Table 2). In basolateral side, the content of quercetin decreased significantly in cells cotreated with BaP, in all time of analysis; the rate of glucuronidation and sulfation of quercetin is increased respect to control, reacting the maximum values at 2h (P<0.01), then both decreased significantly in the last metabolic steps (Table 3). From a comparative analysis of quercetin transport rate in presence of BaP in both cellular compartments (Fig.4) it's possible to observe an increase of glucuronidation rate for each time of analysis but with higher concentrations basolateral than in apical side (Fig 4a). Instead, the sulphatation rate increased in both cellular side until to 2h, with higher concentrations in basolateral than apical sides, and then decreased (Fig 4b). Considering the total conjugates, it showed a similar behaviour in both cellular side: metabolites reacted the highest value at 2h (Fig 4c), considered the maximum metabolic step to possible effects of BaP on phase-2 enzyme, and then decreased slowly until to 8h.

The effects of this co-treatment with BaP were evaluated on the AP:BL efflux ratio, using only quercetin conjugates for the calculation (Fig 5). Quercetin transport rate in presence of BaP was similar to control until 1h and higher from 2h until to 8h, with differences statistically significant (P<0.05). This ratio AP/BL was <1 in all time, showing a basolaterally favoured efflux.

Table 3**:** Quercetin transport rate (conc μM) in basolateral side, in co-treatment with benzo(a)pyrene.

	QUERCETIN	TOT GlcA	TOT Sulphate	TOT Conjugates
T 0	*conc μM*			
QUE (control)	59.99±8.372	-	-	-
QUER (BaP ct)	59.92±9.817	-	-	-
T 30 min	*conc μM*			
QUE (control)	58.422±7.281	0.238±0.032	9.170±1.734	9.409±1.492
QUER (BaP ct)	53.561±8.841	-	11.109±2.341	11.109±2.107
T 1 h	*conc μM*			
QUE (control)	51.963±5.385	0.477±0.054	11.593±1.586	12.363±2.218
QUER (BaP ct)	45.416±8.431	0.973±0.061	12.922±2.073	14.186±2.719
T 2h	*conc μM*			
QUE (control)	31.793±4.387	1.397±0.657	13.732±2.528	15.469±2.781
QUER (BaP ct)	24.012±5.103**	4.997±1.052**	22.542±3.548**	28.053±3.006**
T 4h	*conc μM*			
QUE (control)	18.527±2.527	5.858±1.341	18.863±4.381	25.327±3.718
QUER (BaP ct)	8.951**±1.928	4.267±0.548	14.229±3.205	18.774±2.711
T 8h	*conc μM*			
QUE (control)	2.574±0.822	8.123±1.945	17.237±2.337	25.706±3.106
QUER (BaP ct)	0.255±0.051**	4.699±0.856*	12.798*±1.045	17.713±1.347

P<0.05P<0.01*

DISCUSSION

To assess the fate of food contaminants and phytochemical compounds when they reach the intestinal epithelial cells is very important because their interaction at level of this first barrier either allows or prevents their entry in the gut[34] and, consequently, could influence their toxic and biological effects. In literature several authors report the impact of quercetin and other polyphenols on BaP[41], its toxic activities[42-44], metabolism and enzymatic induction[45-46]. This study, on the contrary, describing the *in vitro* effects of BaP on phase-2 metabolism and transport rate of quercetin confirms that the co-treatment with BaP is able to influence quercetin metabolism, inducing the activity of phase-2 enzyme. Particularly an evident reduction of glucuronidation of quercetin (by *UDP-glucuronosyl transferases*) has been observed in the last time of analysis (from 8h to 24h), while the sulphatation is increased (by s*ulfotransferases*) in the last metabolic rates, in comparison with control. To better understand data obtained, further investigations were carried out to evaluate a possible influence of BaP on quercetin stability but no significant effects were observed in all different experimental conditions of analysis. In fact, the stability of quercetin, analyzed in fresh and spent media, appeared moderately reduced in presence of BaP, respect to control, for all times of analysis. However, in both experimental conditions have to be considered also the slow process of auto-oxidation of quercetin in media. As largely documented by other authors[47], in cell culture the flavonoids decrease intracellular production of reactive oxygen species but, at the same time, may produce them in the extracellular medium, leading to

disorder of redox homeostasis, cellular signaling, transcriptional factors and gene expression[48-49,47].

The transport represents a critical step able to influence the adsorption and metabolism of antioxidants as quercetin and, clearly, the carcinogen BaP is a key factor in the whole process. In fact, the studies carried out in apical and basolateral side of Caco-2 cells, during cotreatment with BaP, showed a clear influence of this toxic compound on transport rate of quercetin and its metabolites. The effect of BaP on transfer rate of quercetin metabolites in both cellular side, with a ratio AP/BL <1 in all time, demonstrated a basolaterally favoured efflux and this is of interest because could favourer also on absorption of this flavonoid.

The increased rate of both glucuronidation and sulfation in apical and basolateral side in presence of BaP could be due to a possible enhancement of proteins transfer or to induction of multi-drug resistance protein transporters[5051], responsible of absorption or excretion of quercetin and its metabolites.

In conclusion, this *in vitro* study showed for the first time that carcinogen B(a)P induced significant changes in phase-2 metabolism and transport rate of the flavonoid quercetin by Caco-2 cells, model of human enterocytes. The influence of BaP on quercetin metabolism and transport rate puts in evidence a possible effect of this toxic compounds as enzymatic inductor of phase-2 enzyme (UDP-GT and ST), probably for their enhancement of enzymatic activities, with significant effects on quercetin bioavailability. However, further investigations are needed to better understand this effects and clarify the real role of BaP on kinetic behaviour of quercetin and, consequently on its biological effects for human health.

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