Research Article

Exposure of breastfed infants to quercetin after consumption of a single meal rich in quercetin by their mothers

Ewa Romaszko¹, Wiesław Wiczkowski², Jerzy Romaszko³, Joanna Honke² and Mariusz K. Piskula²

¹NZOZ Atarax, Olsztyn, Poland
²Department of Chemistry and Biodynamics of Food, Institute of Animal Reproduction and Food Research, The Polish Academy of Sciences, Olsztyn, Poland
³NZOZ Pantamed Sp z o.o., Olsztyn, Poland

Scope: The exposure to quercetin (Q) has not been studied in breastfed infants whose mothers were consuming a Q-rich diet. The objective of the study was to determine whether plant-origin antioxidant-Q passes from the mother’s diet to her milk and to calculate the pharmacokinetic parameters of this phenomenon.

Methods and results: Eleven breastfeeding women were included in this controlled case study. Volunteers followed a Q-restricted diet for 5 consecutive days with the exception of the 3rd day when they received a single meal providing 1 mg of Q per kg of body weight. Urine analysis showed the presence of Q already in the first collected samples after the test (1.5–4 h), which indicated its rapid absorption from the meal. The $C_{\text{max}} = 68 \pm 8.44$ nmol/L concentration of Q in the milk was calculated for $T_{\text{max}} = 11.89 \pm 3.37$ h. It was significantly different ($p = 0.007$) from 40 nmol/L and ($p = 0.016$) from 42 nmol/L of Q concentration before and 48 h after the test, respectively.

Conclusions: Q was shown to be a component of human milk at the nmol/L level. Infants breastfed by mothers consuming a diet rich in Q are exposed to a dose of approximately 0.01 mg of Q daily.

Keywords:
Breastfeeding woman / Human milk / Infants / Onion / Quercetin

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1 Introduction

The composition of human milk is variable; it depends on the infant’s needs (age) and, to a smaller degree, on the mother’s diet [1]. Various types of xenobiotics (contaminants, drugs, secondary plant metabolites) are present in human milk. Their concentration is dependent on the dose ingested by the mother, while other regular components (lipids, proteins, carbohydrates, minerals, etc.) are found in relatively stable concentrations in a given period of breastfeeding [2]. Human milk is also characterized by strictly defined physical and chemical properties, including antioxidant capacity, for which the presence of many enzymatic and nonenzymatic antioxidants is responsible [3–5].

Quercetin, as other polyphenols, exhibits a double face when its influence on the consumer’s health is considered. The most important and complex anti-nutritional effects of polyphenols, including quercetin, are those resulting from their interactions with proteins, which can clearly reduce nutrients’ digestibility through inhibition of proteolytic, lipolytic, and glycolytic enzymes leading to lowering of nutrients’ assimilation. There are also observed problems with mineral deficiency related to the formation of complexes between...
polyphenols and metal cations that interfere with intestinal absorption of metals, especially for iron, calcium, and zinc. The above problems are extremely important when nutrition of breastfed infants is considered.

Quercetin (Q) is one of the best-described plant origin antioxidants and this ability is associated with the health-protecting effect of consumption of flavonoids with fruit and vegetables, e.g. onions, apples, grapes. Q is rapidly absorbed from the gastrointestinal tract and it is metabolized via a set of P II detoxification processes including glucuronidation, sulfation and methylation, producing over 20 different metabolites retaining a native structure of Q [6–8] and these eliminated with urine form approximately 2–3% of the ingested dose [8, 9]. Moreover, flavonoids’ metabolite concentration in urine shows a close correlation with those in the serum [10, 11].

Bioavailability of flavonoids is influenced by a number of factors; solubility of a compound in the digestive tract, nutritional status of a subject (fasted or not fasted) or the chemical form in which the compound was ingested (glycosides or aglycone) are the examples [12].

When analyzing Q bioavailability, its dependence on the dietary matrix and chemical form in which it was ingested (glycosides or aglycone) as well the phenomenon of its enterohepatic circulation have to be considered. The biological half-life of Q is relatively long (11–28 h), which means that with regular intake the compound may accumulate and reach steady-state concentration [9, 11, 13]. Animal studies suggest that large quantities of Q accumulate in the lungs and in smaller quantities in the brain, adipose tissue and the spleen [14–16]. Concerning the mammary gland tissue, only the presence of isoalvones was reported in ewes [17]. No data are available on the presence of Q in breast tissues or in human milk and there are very few studies showing the presence of phenolic compounds other than Q in the breast tissue. Till now, special attention has been paid to nutritional exposure of breastfed infants to phytoestrogens via milk of mothers consuming soy products or via milk formulas based on soy proteins. The occurrence of phytoestrogens (daidzein and genistein), chalcones (xanthohumol), and flavones (isoxanthohumol) of which the concentration depended on the dose ingested [18–20] in the human breast milk has been already reported.

Ingested food component can act locally, at the level of the digestive tract or after absorption at the systemic level. Nevertheless, there is very little data on absorption of phytoestrogens taken with mother’s milk by infants. In a study of Cao et al. [21] soy phytoestrogens were undetectable in infants’ blood; however some were found in the urine, indicating that their absorption occurs. Nevertheless, the implication of this absorption on newborns’ development is still not known.

Based on its chemical structure, it can be expected that Q may also be present in human milk [22]. This study is the first attempt to assess Q content in human milk along with the pharmacokinetic parameters after the mothers’ test with Q rich meals and estimation of infants’ exposure to this xenobiotic.

2 Materials and methods

2.1 Subjects

The study was registered and approved by the Bioethics Committee of the Warmian-Mazurian Chamber of Medical and Dental Professionals in Olsztyn, Poland as of 25/2008/III. The subjects were recruited for the study through an advertisement at Family Out-Patient Clinics. The infants had to fall within the age range from the end of the 1st month to the end of the 10th month of life. Mothers on long-term treatment with any medications, following selective diets (e.g., a vegetarian diet), drinking coffee, alcohol, or with any known food allergy or with obesity (BMI ≥ 30 kg/m²) were excluded from the study. An informed consent from both parents was obtained after providing them with oral and written information about the study. A total of 11 healthy mother–infant pairs were included in this open-label experiment.

2.2 Study design and diets

The subjects agreed to follow a low-Q diet for 5 consecutive days. They were provided with a list of food products to avoid including: onions and onion-containing products, broccoli, raspberries, buckwheat groats, green tea, herbal teas, grapes, cabbage, wine, coffee, chocolate, cocoa, honey, apples, lettuce, tomatoes, and green beans.

For three days of the Q restricted diet mothers were asked to collect samples of milk (3–5 mL) and urine (2 × 10 mL) into the labeled tubes every 12 h, to mark the collection time and to immediately freeze them at −20°C. Next, they were given, on a one-off basis, a standardized Q-rich meal following which they continued the Q-restricted diet until the end of the fifth day of the study.

Milk and urine samples were regularly collected in the same manner. However, milk was sampled at a prespecified schedule (3, 6, 12, 18, 24, 36, and 48 h postexposure) and urine as two 24-h collections. For urine samples the mothers received graduated cylinders to measure the total volume of collected urine and tubes for 2 × 10 mL urine samples from each collection with the labels to note the time and total volume of the urine collected. Both milk and urine samples were frozen at −20°C immediately after collection. Frozen milk and urine samples were collected from the mothers and transported in the frozen state to the laboratory deep freezers on a daily basis, and kept there at −80°C until analyzed, but not longer than 3 months. All the mothers breastfed “on demand” and kept a breastfeeding diary for 5 days. There were no restrictions as to the place of collection. However, contact with the mothers was maintained to arrange the pickup of the frozen samples.

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The Q-rich meal was onion soup prepared from poultry stock to which freeze-dried pulverized parenchyma scales of the yellow onion with a predetermined Q content were added in such a proportion that one portion of the onion soup, of which the volume was calculated on the basis of the mother’s body weight provided about 1 mg of Q per kg of body weight. Because the soup had to be freshly prepared, it was technically impossible to determine the actual concentration of Q directly before the soup was served. For this reason, each time the soup was cooked, two 200 mL samples were collected and two further samples were collected after the soup was consumed by the last mother on that day and frozen at −80°C until analysis. Apart from biological sample collection and analysis, a detailed record of individual feedings, which included baby behaviors during feeding on-demand or which breast was preferred by babies, was run.

2.3 Determination of quercetin in the experimental meal

The analytical procedures were based on the method described by Wiczkowski et al. [23]. Briefly, it involved the extraction of Q from the matrix and its analysis by HPLC-PDA-MDESI (Shimadzu system; HPLC 10V AD with Q-alfa 8000 mass spectrometer). The samples of the freeze-dried soup (about 100 mg) were subjected to quintuple extraction with 1 mL of 80% methanol v/v by sonication and vortexing. Q and its glucosides were analyzed with UV detection at 360 nm with the flow rate of mobile phase 0.2 mL/min on a C18 Cadenza 5–μm column, 250 × 2 mm (Imtak, USA) at 45°C. The analytes were eluted in a gradient system where phase A consisted of water/TFA, 995/5 v/v, phase B consisted of acetonitrile/TFA, 995:5 v/v and concentration gradient of 21–80–80–21–21% of phase B in the time gradient of 0–27–32–35–55 min was used. The compounds were identified on the basis of the values of their molecular ions and retention times of external standards. For quantitative analysis the calibration curve with the range of 0.2–50 μM was used.

2.4 Determination of quercetin in the milk and urine

2.4.1 Nonconjugated quercetin in the milk

The milk samples were thawed, 12.5 μL/mL of 1.0 M ascorbic acid in water (protective antioxidant) was added and after mixing, depending on the initial milk sample volume, they were divided into 0.6–0.8 mL portions followed by addition of 15 μL of 10 μM fisetin methanol solution to each portion as the internal standard. In order to remove fat the entire mixture was subjected to duplicated extraction with 3 mL of hexane by 30-s vortexing and sonication followed by centrifugation at 12 000 × g for 10 min. After each centrifugation the upper fat-containing layer was collected with Pasteur pipette and discharged. Finally, after the second extraction the remaining hexane was removed with the stream of nitrogen and the defatted milk was mixed with an equal volume of 0.2 M acetic acid buffer (pH 5.0) and subjected to Q analysis as below.

2.4.2 Nonconjugated quercetin in the urine

After thawing the urine, 50 μL samples were mixed with 50 μL of 60 mM ascorbic acid water solution, 10 μL of 50 μM fisetin methanol solution as the internal standard and with equal volume of 0.2 M acetic acid buffer (pH 5.0) and subjected to Q analysis as below.

2.4.3 Conjugated quercetin metabolites in the milk and urine

To analyze the total absorbed Q content (both nonconjugated and conjugated), the hydrolysis of the Q and isorhamnetin (methylated metabolite of Q) conjugates was necessary. For this purpose 0.2 M acetic acid buffer (pH 5.0) containing β-glucuronidases (1000 U/mL) and sulfatases (3.5 U/mL) was added at equal volumes to the defatted milk or urine containing the internal standard and ascorbic acid. This solution was prepared from the mixture (6:1; U/U) of sulfatase H–1 from Helix Pomatia and sulfatase H–VI from Aerobacter aero.
genesis (Sigma, USA). It is worth mentioning that sulfatase H-1 apart from its sulfatase activity has a strong secondary activity of β-glucuronidase like 1U:300U, respectively. The resulting solutions were incubated at 37°C on the water-shaking bath for 1 h. Next, to stop the enzymatic reaction and to extract the released Q and isorhamnetin, 1 mL of ethyl acetate was added. The resulting mixture was subjected to 30-s vortexing and 30-s sonication followed by centrifugation at 12 000 × g for 20 min. This was done in triplicate, each time collecting the ethyl acetate layers which, after combining, were evaporated until dry in a nitrogen stream. The residue was dissolved in 100 μL of a methanol/TFA mixture (995:5; v/v), centrifuged at 12 000 × g for 20 min and the volume of 10 μL injected for analysis by HPLC-PDA-MS-ESI as described above. The results were corrected for the internal standard recovery factor that was within the range of 1.12–1.21 for urine and 1.19–1.40 for milk. For quantification, the calibration curve for urine and milk with the range of 0.10–30.00 μM was used.

2.5 Pharmacokinetic parameters of quercetin in the human milk

Based on Q concentrations in the breast milk the following pharmacokinetic parameters were calculated: C_{max}—peak Q concentration between 0 and 48 h postexposure; T_{max}—time to peak Q concentration in the milk between 0 and 48 h postexposure; the area under the curve Q concentration in milk versus time during 0 and 48 h postexposure; K_{el}—Q elimination rate; T_{1/2}—Q half-life in the milk; Q mean residence...
time. The calculations were done with the Biokinetica 3.1 software, with the trapezoidal rule method. The parameters were calculated for each subject.

2.6 Statistical analysis

Concentration of quercetin and isorhamnetin in urine as well pharmacokinetic parameters are expressed as the mean of individual measurements for each subject ± SEM. The results of quercetin and isorhamnetin determination in the milk for each subject were checked to see if they had normal distribution (Kolmogorov–Smirnov test) and if assumptions of variance equation were met (Levene test). Comparison of the mean concentrations values of Q and IR in the milk before and after the mothers’ exposure to Q-rich meals was done with the t-test for dependent variables. Differences were considered significant when p < 0.05.

3 Results

Eleven breastfeeding women, aged 25–40, and their 11 healthy, thriving infants participated in the study. All babies were born at term with Apgar scores 8–10. The mean of birth weight was 3.52 ± 0.35 kg. The mean age of the mothers participating in the study was 32.00 ± 2.36 years. The mean age of the infants was 7.21 weeks.

Table 1. Quercetin (Q) content in soup samples in mg per 100 mL of the soup

<table>
<thead>
<tr>
<th>Soup</th>
<th>Q-3,4′-glucoside</th>
<th>Q-3-glucoside</th>
<th>Q-4′-glucoside</th>
<th>Isorhamnetin-4′-glucoside</th>
<th>Q</th>
<th>Total Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soup 1</td>
<td>5.21</td>
<td>0.17</td>
<td>11.11</td>
<td>0.80</td>
<td>0.42</td>
<td>17.85</td>
</tr>
<tr>
<td>Soup 2</td>
<td>7.04</td>
<td>0.23</td>
<td>14.91</td>
<td>1.12</td>
<td>0.58</td>
<td>23.31</td>
</tr>
</tbody>
</table>

a) Presented values are the means from duplicate analysis. Values for glucosides are expressed in quercetin aglycone.

Figure 2. Quercetin concentration in the subjects’ breast milk. Means ± SEMs; Values labeled with the same letter are significantly different (p < 0.05).

usually in the 3rd hour (from 1.5 to 6 h) after consumption to decrease to the levels from before the test 48 h later. The method used here for Q metabolites determination is based on the enzymatic treatment of milk or urine with enzymes capable of hydrolyzing only glucuronide and sulfate conjugates, therefore as a result of this treatment only Q and its methylated derivative—isorhamnetin are produced and they represent the total metabolites of ingested Q. It is worth noting that among the Q metabolites determined in the urine, isorhamnetin accounted for 13.56–29.03%. Having the volumes of urine with respective Q concentrations, it was possible to calculate that within the first 24-h postexposure the volunteers eliminated between 1.09 and 3.42% of the ingested dose in their urine, and the most rapid elimination and the highest concentrations were observed within the postexposure 1.5–6 h. Individual profiles of urine Q concentration are presented in Fig. 1 where high individual variations can be noticed (figure with full range of observations is available online as supporting information).

Since the volume of the milk produced by the mammary glands of the mothers was not measured, the data obtained from the milk analysis only allowed us to measure the concentrations and not the quantities of the investigated substances. Of note were the differences in the concentrations of Q in the milk compared to those in the urine, with the former ones being expressed in nmol/L, three orders lower than those in the urine. Despite the interindividual differences, the temporal profile of mean concentrations of Q in milk (Fig. 2) is a good reflection of the curves shapes obtained for individual mothers. The calculated pharmacokinetic parameters of Q in the milk are provided in Table 2. The mean Q concentration in
the first milk samples collected from the subjects at the start of the Q-poor diet was 45 nmol/L (range: 19–102 nmol/L). Following the test meal consumption the time to reach peak Q concentration in the milk ($T_{\text{max}}$) differed between the subjects. Although the first increases in Q concentration were observed already within 3–6-h postexposure, the $T_{\text{max}}$ and $C_{\text{max}}$ values calculated from the mean values were $11.89 \pm 3.37$ h and $68 \pm 8.44$ nmol/L, respectively. $C_{\text{max}}$ was significantly higher ($p < 0.05$) than mean Q concentration from the last sampling ($t = -10$ h) before the exposure ($c = 40$ nmol/L) as well as significantly higher ($p < 0.05$) than the mean value at last postexposure sampling ($t = +48$ h) that was $c = 42$ nmol/L.

Concerning feeding frequency, the babies were breastfed an average of $8.26 \pm 2.26$ times a day, and any given baby could be fed 8 times on one day and 12 times on another, could not be fed one night at all and twice another. There was no significant indication which breast was preferred.

4 Discussion

4.1 Quercetin absorption and transport

Onion soups offered to subjects slightly differed in Q concentrations (Table 1) which probably resulted from Q thermal degradation [24]. Due to relatively low Q bioavailability in humans these differences were regarded as insignificant for the study. Q is readily absorbed from the human gastrointestinal tract and reaches its peak concentration in the plasma within 0.5 to 6.0 hours following ingestion, depending on its chemical form (glycosides or aglycone) and the food matrix [25–27]. After ingestion, Q glucosides may be transported directly to enterocytes by a sodium-dependent glucose transporter SGLT1 [28]. However, a recent investigation questions this mechanism [29]. When Q is present in food as an aglycone, due to its hydrophobic coplanar structure it can pass into enterocytes via passive transport. In intestinal epithelial cells, Q is subjected to the action of phase-II enzymes forming glucuronides, sulfates and/or methylated metabolites [24]. A portion of metabolites’ pool enters the circulatory system and is transported via the portal vein to the liver where further phase II metabolism can take place. Q metabolites produced in the enterocytes may be returned to the gastrointestinal tract. Several studies have demonstrated that multidrug resistance-associated protein-2 participates in this phenomenon [30]. Q metabolites may be removed from the circulatory system through the liver with bile back into the gastrointestinal tract. They can be in part reabsorbed from the colon, thus forming enterohepatic circulation. Metabolites that escape from these efflux systems are distributed via blood circulation as complexes with albumin and are finally excreted into the urine and, as it follows from our results presented here, also into the milk.

In the mammary glands five transport routes of proteins, lipids, ions, nutritional substances, and water for milk have been described: exocytosis, membrane transport, lipid excretion route, transcytosis, and paracellular transport. The first four pass through the cells and include transport through membrane barriers, and the last one, paracellular, provides

<table>
<thead>
<tr>
<th>$T_{\text{max}}$ [h]</th>
<th>$C_{\text{max}}$ [nmol/L]</th>
<th>AUC [nmol/L × h]</th>
<th>$T_{1/2}$ [h]</th>
<th>$K_{\text{el}}$ [h⁻¹]</th>
<th>MRT [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$11.89 \pm 3.37$</td>
<td>$68 \pm 8.44$</td>
<td>$2609.68 \pm 224.15$</td>
<td>$50.33 \pm 9.79$</td>
<td>$0.014 \pm 0.003$</td>
<td>$22.35 \pm 0.87$</td>
</tr>
</tbody>
</table>

AUC, area under the curve; MRT, mean residence time.
a direct component exchange between the follicular lumen and interstitial space [31]. The results of studies on humans demonstrate expression of xenobiotics transporters in the mammary glands during lactation and prove their vital role in the accumulation of some compounds in milk. Molecular characteristics of these transporters show their considerable diversification; they have been identified also as the transporters belonging to the ABC group and playing a key role in the transport of flavonoid conjugates through epithelial cells, especially glycoprotein P and multidrug resistance-associated protein-2 [32–34].

This study provides no ground for indicating which mechanism is relevant in Q transport to milk. However, based on the assumption that large-molecule substances are excreted to milk with transcytosis, albumin complexes with Q metabolites are potential candidates for this mechanism. Another option is based on the fact that Q occurs in the blood circulation in the form of metabolites (glucuronides, sulfates, and methylated derivatives) with increased hydrophilicity compared to native Q. Terao et al. claim that flavonoids have intermediate properties (hydrophilic–hydrophobic) due to which they can be localized on the border between two phases (water–lipid) [35]. Taking into account the long half-time and late maximum concentration in blood, it can be presumed that Q metabolites may pass to milk by passive diffusion [36].

4.2 Quercetin content in urine and milk

In this study, Q was found in all the urine and milk samples. The ingestion of a Q-rich meal resulted in a significant increase in the concentrations of its metabolites in the mothers’ urine and milk (Figs. 1 and 2). The concentration of Q metabolites in the urine is both a marker of the efficacy of the restrictive diet and of the loading dose. Due to ethical issues, we decided not to determine Q concentrations in the mothers’ blood plasma bearing in mind that urine and plasma Q concentrations show temporal correlation [37, 38]. During the Q restricted diet up to the exposure to this compound, there was no significant decrease in Q concentration in the mothers’ milk (Fig. 2). Maintaining a certain low Q content in the milk, in spite of the mothers being on a diet reducing Q consumption, suggests a possibility of accumulating this compound in the organism, perhaps also in the mammary glands. It may also be the effect of long Q half-time, which in this study was found to be 50.33 h and mean residence time of 22.35 h (Table 2), or a result of the enterohepatic circulation of Q, or simply mothers’ not following the restrictions of Q containing food consumption. Yet, after the mothers’ exposure to Q a statistically significant increase in its concentration in the milk was followed by a drop in its concentration down to preexposure level.

4.3 Exposure of infants to quercetin via mothers’ milk

An estimate of the exposure of the breastfed baby to Q was calculated assuming that a baby at 24.82 ± 7.21 weeks consumes about 900 mL of milk daily [44]. Given that the mean Q concentration in the human milk was 45 nmol/L, the total daily Q dose could be estimated at 0.01 mg. This is in a very good agreement with Setchell et al. [45], who determined this value for phytoestrogens taken by the breastfed infants whose mothers consumed 20–25 mg of isoflavones as soy products at 0.005–0.01 mg.

In his study, Meyer [46] fed pregnant rats with a preparation consisting in 90% of diosmin and 10% of other flavonoids and determined fetus exposure to flavonoids. Diosmin was found in the fetus (0.003% of the dose) and in the mother’s milk (up to 1% of the dose). If the rat data can be at least partly related to humans, it can be a contribution to the assumption that babies are already exposed to flavonoids in the fetal stage of life and the exposure is continued throughout the breastfeeding period.

The influence of polyphenols on infants’ health is more important when the digestive tract level is considered. First, their impact on development and composition of gut microbiome is already proven [47] and this is directly linked with the newborns’ gut immune system modulation [48, 49]. The results presented here prove that Q is present in the milk of mothers consuming plant-derived foods and its concentration increases following consumption of foods rich in Q.
which was to date shown only for isoflavones, though, without quantitative postprandial relation. Therefore, infants’ demonstrated exposure to quercetin can have an influence on their health via nutritional and developmental actions.

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5 References


