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Original Article

Quercetin uptake and metabolism by murine peritoneal macrophages *in vitro*

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ARTICLE INFO

Article history:

Received 11 April 2014

Received in revised form

11 June 2014

Accepted 12 June 2014

Available online 10 January 2015

Keywords:

metabolism

mouse peritoneal macrophages

quercetin

uptake

ABSTRACT

Quercetin (Q), a bioflavonoid ubiquitously distributed in vegetables, fruits, leaves, and grains, can be absorbed, transported, and excreted after oral intake. However, little is known about Q uptake and metabolism by macrophages. To clarify the puzzle, Q at its noncytotoxic concentration (44 μ M) was incubated without or with mouse peritoneal macrophages for different time periods. Medium alone, extracellular, and intracellular fluids of macrophages were collected to detect changes in Q and its possible metabolites using high-performance liquid chromatography. The results showed that Q was unstable and easily oxidized in either the absence or the presence of macrophages. The remaining Q and its metabolites, including isorhamnetin and an unknown Q metabolite [possibly Q⁻ (O-semiquinone)], might be absorbed by macrophages. The percentage of maximal Q uptake by macrophages was found to be 2.28% immediately after incubation; however, Q uptake might persist for about 24 hours. Q uptake by macrophages was greater than the uptake of its methylated derivative isorhamnetin. As Q or its metabolites entered macrophages, those compounds were metabolized primarily into isorhamnetin, kaempferol, or unknown endogenous Q metabolites. The present study, which aimed to clarify cellular uptake and metabolism of Q by macrophages, may have great potential for future practical applications for human health and immunopharmacology.

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1. Introduction

Quercetin (Q), a typical antioxidative flavonoid ubiquitously distributed in vegetables, fruits, leaves, and grains, can be absorbed, transported, and excreted after oral intake, suggesting that cellular uptake of quercetin aglycone and quinine formation are possible [1]. An aglyconic version of Q can be

found in the blood after the digestion of quercetin glycosides via the small intestine, although Q may conjugate with different glycosides to form quercetin glycosides in plants. After Q supplementation, levels of Q and its methylated metabolite isorhamnetin in the plasma and brain increase markedly, further indicating Q uptake and metabolism *in vivo* [2]. Q, in its aglyconic form, can transiently reach any blood

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<http://dx.doi.org/10.1016/j.jfda.2014.06.011>

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cells including immune cells *in vivo*. However, little is known about Q uptake and metabolism by macrophages.

Macrophages are the mature form of monocytes resident in almost all tissues and are pivotal cells in innate immunity. Macrophages are relatively long-lived and versatile cells that perform several different functions throughout the innate immune response and subsequent adaptive immune responses [3]. It is clear that monocytes and macrophages are vital to immune response regulation and inflammation development. Q was found to significantly reduce the production of proinflammatory cytokines interleukin-6 and tumor necrosis factor- α in THP1 macrophages [4]. Recently, Q-rich extracts from strawberry and mulberry fruits were found to significantly decrease mouse splenocytes' tumor necrosis factor- α /interleukin-10 (pro-/anti-inflammatory) cytokine secretion ratios in the presence of lipopolysaccharide (LPS) in concentration-dependent manners [5]. Most recently, it was found that Q administered in a prophylactic manner might act as an immunostimulatory agent; however, Q exhibited a therapeutic, but not prophylactic, effect on spontaneous or LPS-induced inflammation *in vivo* [6]. Taken together, Q has been considered a potent bioflavonoid, and widely used in health foods and pharmacology. Undoubtedly, Q may affect immune cells. However, only a few reports regarding research on Q uptake and metabolism by macrophages were found.

Q was found to have great immunomodulation potential *in vitro* and *in vivo*. We hypothesized that macrophages might have a specific mechanism to Q uptake and metabolism. To validate this assumption, Q was incubated with mouse peritoneal macrophages for different time periods. Extra- and intracellular fluids from the cultures were collected. Changes in the levels of Q and its possible metabolites were measured using high-performance liquid chromatography (HPLC) to clarify Q uptake and metabolism. The present study to clarify cellular uptake and metabolism of Q by macrophages may have great potential for its future practical applications for human health and immunopharmacology.

2. Materials and methods

2.1. Chemicals

Quercetin dihydrate ($C_{15}H_{10}O_7 \cdot 2H_2O$; 338.27 g/mol; Sigma-Aldrich Co., Steinheim, Switzerland) was purchased at the highest available purity (>98%, HPLC) and dissolved in dimethyl sulfoxide (Wako, Osaka, Japan) to prepare a stock solution at a concentration of 20mM. The stock solution was sterilized using a filter (Millipore, Carrigtwohill, Cork, Ireland) with 0.22 μ m pore size and stored at -80° C for future use. Q, rutin hydrate, ellagic acid, *p*-coumaric acid, morin, kaempferol (Sigma-Aldrich Co., Steinheim, Germany), and isorhamnetin (MP Biomedicals, Illkirch, France) were purchased at the highest available purity (>98%, HPLC) for HPLC standards.

2.2. Source of mouse primary peritoneal macrophages

Female BALB/cByJNarl mice (6-week old) were obtained from the National Laboratory Animal Center, National Applied

Research Laboratories, National Science Council in Taipei, Taiwan, R.O.C., and maintained in the Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan, R.O.C. The mice were housed and kept on a chow diet (laboratory standard diet, Diet MF 18; Oriental Yeast Co., Ltd., Osaka, Japan). The animal room was kept on a 12-hour-light and 12-hour-dark cycle. Constant temperature ($25 \pm 2^\circ$ C) and relative humidity (50–75%) were maintained. After the mice were acclimatized for 4 weeks, they were sacrificed to obtain peritoneal macrophages. BALB/c strain mice weighing 20–25 g were used throughout the experiment. The animal-use protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee, National Chung Hsing University.

The primary peritoneal macrophages from mice were collected according to the method described by Lin and Tang [7] and Liu and Lin [8]. Briefly, the adult female BALB/c mice were anesthetized with diethyl ether, bled using a retro-orbital venous plexus puncture to collect blood, and immediately euthanized by CO₂ inhalation. Peritoneal macrophages were prepared by lavaging the peritoneal cavity with two aliquots of 5 mL sterile Hank's balanced salts solution [50 mL of 10 \times Hank's balanced salts solution (Hyclone Laboratories Inc., Logan, UT, USA); 2.5 mL of antibiotic–antimycotic solution (100 \times penicillin-streptomycin-amphotericin (PSA)) containing 10,000 units of penicillin, 10 mg streptomycin, and 25 μ g amphotericin B per milliliter in 0.85% saline (Atlanta Biologicals Inc., Norcross, GA, USA); 20 mL of 3% bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄, 1.5mM KH₂PO₄, pH 7.4, 0.2 μ m filtered); 2.5 mL of 7.5% NaHCO₃ (Wako), and 425 mL sterile water], for a total of 10 mL, through peritoneum. The peritoneal lavage fluid was centrifuged at 400 \times g for 10 minutes at 4°C. The cell pellets were collected and resuspended in a tissue culture medium (TCM, a serum replacement; Celox Laboratories Inc., Lake Zurich, IL, USA), suspended in a medium consisting of 10 mL TCM, 500 mL Roswell Park Memorial Institute 1640 medium (Atlanta Biologicals Inc.), and 2.5 mL antibiotic–antimycotic solution (100 \times PSA). The peritoneal adherent cells (>90% of macrophages) from each animal were pooled and adjusted to 2 \times 10⁶ cells/mL in TCM medium with a hemocytometer using the trypan blue dye exclusion method.

2.3. Effect of Q treatment on macrophages cell viability

To evaluate the possible cytotoxic effect of Q, the macrophage cell viability was determined using a 3-(4,5-dimethylthiazol-2-diphenyl)-2,5-tetrazolium bromide (MTT) assay. Briefly, peritoneal macrophages (2 \times 10⁶ cells/mL in TCM, 50 μ L/well) in the absence or presence of Q (88 μ M in TCM, 50 μ L/well) were cocultured in 96-well plates at 37°C in a humidified incubator with 5% CO₂ and 95% air for 24 hours. Aliquots of 10 μ L of 5 mg/mL MTT (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS were added to each well in the 96-well plate. The plates were incubated for another 4 hours. The culture medium was then discarded. The plates were carefully washed with PBS buffer twice. Aliquots of 100 μ L of dimethyl sulfoxide were added to each well and oscillated for 30 minutes to extract the formed insoluble formazan. The absorbance was measured at 550 nm

on a plate reader (ELISA reader, ASYS Hitech, GmbH, Eugendorf, Austria). The cell viability of macrophages was calculated using the absorbance (A) at 550 nm:

$$\text{Cell viability (\% of control)} = [(A_{\text{sample}} - A_{\text{sample blank}})/(A_{\text{control}} - A_{\text{blank}})] \times 100. \quad [1]$$

The remaining cell viability showed that Q treatment at a final concentration of 44 μM did not have any cytotoxic effect on peritoneal macrophages. Thus, 44 μM Q (= 15 $\mu\text{g}/\text{mL}$) was adopted for use in the following cellular uptake and metabolism experiments.

2.4. Determination of Q uptake and metabolism in macrophages

To measure Q uptake and metabolism in mouse peritoneal macrophages, Q (88 μM , 100 $\mu\text{L}/\text{well}$), in the absence or presence of mouse peritoneal macrophages (2×10^6 cells/mL, 100 $\mu\text{L}/\text{well}$), were cocultured in 96-well plates. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for different time periods. After incubation for a given time, the incubated fluid was centrifuged at 4°C, $400 \times g$ for 10 minutes. Aliquots of 200 μL of fluid in medium alone and cell culture supernatants (namely, extracellular fluids) were collected and stored in the dark at –80°C for measuring individual flavonoid contents. The cell pellets were carefully washed with PBS buffer twice, lysed, and shaken in the dark at 4°C for 10 minutes with 200 μL of lysis buffer (pH 7.4) containing 10 mL of 10mM tris(hydroxymethyl)aminomethane (Tris, pH 7.4), 15 mL of 150mM NaCl, 10 mL of 5mM EDTA, 10 mL of 1mM sodium orthovanadate, 5 mL of 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride, 6 mg of 4 trypsin inhibitor unit/mg aprotinin, 1 mL of 0.1% sodium dodecyl sulfate, and 1 mL of Triton X-100 in 100 mL solution [8]. The mixture was allowed to stand at 4°C for another 30 minutes. The resulting mixtures were collected into an Eppendorf tube and centrifuged at 4°C, $16,000 \times g$ for 20 minutes. The supernatants, namely, intracellular fluids, were collected and another 8 mL PBS was added. The fluids were stored in the dark at –80°C prior to determining individual flavonoid contents.

To extract total flavonoids from the samples, the sample was added with an equal aliquot volume of 2M hydrochloric acid (Wako) to precipitate the protein and increase flavonoid recovery. The mixture was shaken and incubated in the dark at room temperature for 30 minutes. After incubation was completed, an aliquot of 15 mL ethyl acetate was added and the mixture was shaken for another 3 minutes. The resultant mixture was centrifuged at $400 \times g$ for 10 minutes at room temperature. The top supernatant (about 15 mL) was collected, and the bottom solution was added another 15 mL ethyl acetate. The mixture was shaken for another 3 minutes and centrifuged at $400 \times g$ for 10 minutes at room temperature. The top supernatant (about 15 mL) was collected and pooled. The obtained ethyl acetate solution was mixed, and the solvent was removed using a rotary vacuum evaporator (EYELA; Tokyo Rikakikai Co., Ltd, Bohemia, NY, USA). The residues were resuspended in 5 mL deionized water and 200 μL ethyl acetate. The extracts were lyophilized and resuspended in 0.5 mL of methanol (HPLC-grade; Tedia Co.

Inc., Fairfield, OH, USA) and filtered through a 0.2 μm filter (Minisar SRP4, PTFE membrane; Sartorius, Goettingen, Germany). The filtrate sample was stored in the dark at –80°C until use.

2.5. HPLC analysis of individual flavonoids

The sample solution was ultrasonically degassed prior to HPLC analysis. Mobile phase A [double-distilled water:tetrahydrofuran (ECHO, Miaoli, Taiwan):trifluoroacetic acid (Sigma-Aldrich Co., St. Louis, MO, USA) = 98:2:0.1, v/v/v] and mobile phase B (acetonitrile, HPLC grade; ECHO) were filtered through a 0.45 μm filter (Durapore, Millipore, MA, USA) under vacuum and ultrasonically degassed prior to use. The mobile phase flow rate was 1 mL/min. Aliquots of 20 μL sample solution were subjected to HPLC analysis. A pump (L-2131; Hitachi, Tokyo, Japan), a UV-visible detector (L-2400; Hitachi), and a chromatographic separation column ($250 \times 4.6 \text{ mm}^2$, 5 μm ; Mightsil RP-18 GP250; Kanto Chemical Co, Inc., Tokyo, Japan) were used. The pump was controlled under the D-2000 Elite program (Hitachi): a gradient elution starting at 83% mobile Phase A and 17% mobile Phase B for 7 minutes, followed by 75% mobile Phase A and 25% mobile Phase B for 8 minutes, 65% mobile Phase A and 35% mobile Phase B for 5 minutes, 50% mobile Phase A and 50% mobile Phase B for 7 minutes, 0% mobile Phase A and 100% mobile Phase B for 8 minutes, and ending at 83% mobile Phase A and 17% mobile Phase B for 5 minutes. The detection was at 370 nm [5]. Seven compounds, including rutin, ellagic acid, *p*-coumaric acid, morin, Q, kaempferol, and isorhamnetin, were selected as standards. The retention times (RTs) for rutin, ellagic acid, *p*-coumaric acid, morin, Q, kaempferol, and isorhamnetin were found at 10.4 minutes, 10.8 minutes, 11.4 minutes, 17.8 minutes, 19.7 minutes, 23 minutes, and 23.3 minutes, respectively [5]. Individual compound quantification levels were based on the peak integral area ratio between the standard and sample. To compare changes in Q levels in extracellular fluids, a relative uptake percentage was calculated using the following equation:

$$\text{Relative percentage of Q content (\%)} = (\text{residual content of Q at given incubation time}/\text{added original content of Q}) \times 100. \quad [2]$$

In intracellular fluids, a relative percentage of Q content was calculated using the following equation:

$$\text{Relative percentage of Q content (\%)} = [\text{residual content of Q at given incubation time}/(\text{added original content of Q} - \text{residual content of Q in extracellular fluid at the given incubation time})] \times 100. \quad [3]$$

The relative percentage of Q content in intracellular fluids was defined as the percentage of Q uptake by the cells.

2.6. Statistical analysis

Data were analyzed using the Windows SAS program (Version 8.0, SAS Institute Inc., Cary, NC, USA). Values were expressed

as means \pm standard error of the mean ($n = 6$ biological determinations) using analysis of variance, if justified by the statistical probability ($p < 0.05$), followed by Duncan's new multiple range test. Differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

We hypothesized that macrophages might take up Q and further metabolize it into different metabolites. To confirm this assumption, Q was incubated in the absence or presence of mouse peritoneal macrophages for different time periods.

3.1. Effect of Q treatment on macrophage cell viability

In our previous study, we found that Q treatments at final concentrations $< 50\mu\text{M}$ did not produce cytotoxic effects on the macrophages [6]. To avoid unpredictable cytotoxicities at higher doses, an aliquot of $44\mu\text{M}$ Q was selected to reconfirm the macrophage cell viability, indicating that $44\mu\text{M}$ Q did not have any cytotoxic effect on macrophages (Fig. 1). Based on the results, $44\mu\text{M}$ Q ($= 15\mu\text{g/mL}$) was selected for use in the following cellular uptake and metabolism experiments. In addition, a bioavailability study on Q in rats fed 0.125%, 0.25%, and 0.50% Q diet for 10 days reflected $45 \pm 6\mu\text{M}$, $79 \pm 7\mu\text{M}$, and $118 \pm 8\mu\text{M}$ of plasma Q concentrations, respectively [9]. Evidently, $44\mu\text{M}$ Q selected for use in the present study is safe and possible to reach *in vivo*.

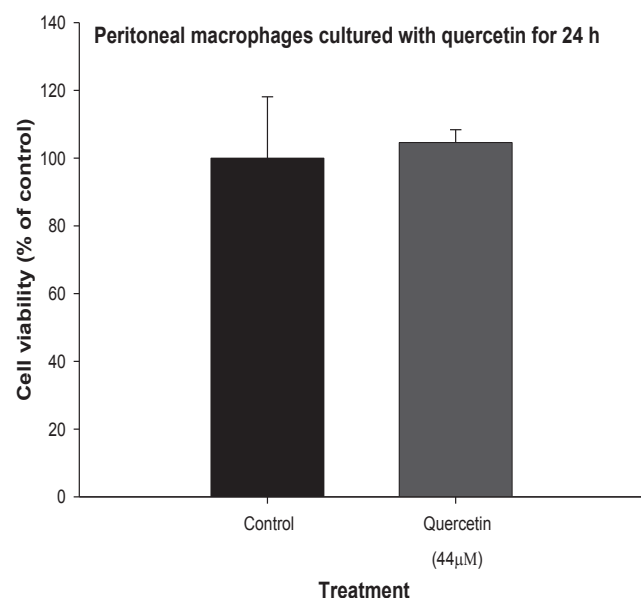


Fig. 1 – Effect of quercetin treatment on the macrophage cell viability. Values are expressed as means \pm SEM ($n = 6$ biological determinations), analyzed using one-way ANOVA, followed by Duncan's multiple range tests. There are no significant differences between quercetin treatment and the control. The original cell density was 1×10^6 cells/mL. ANOVA = analysis of variance; SEM = standard error of the mean.

3.2. Changes in levels of Q and its metabolites in medium alone or macrophage extracellular fluid treated with Q through different incubation times

Changes in Q and its metabolites in medium alone (Fig. 2) and extracellular fluids of macrophages cultured with Q for different time periods (Fig. 3) are shown as HPLC chromatograms. Levels of Q and its metabolites in medium alone and extracellular fluids of macrophages incubated for different time periods were analyzed according to their HPLC chromatograms. The results showed that Q levels in medium alone decreased markedly as the incubation time was extended, indicating that Q was unstable and readily oxidized itself (Fig. 4A). Accordingly, isorhamnetin (3'-O-methyl quercetin; Fig. 4A), kaempferol, unknown Q metabolites (UQMs, RT: 20.7 minutes and 25 minutes), and other trace metabolites (RT: 7.6 minutes; Fig. 4B) were found. Importantly, the relationship between Q and isorhamnetin showed a negative correlation as the incubation time was extended, suggesting that isorhamnetin might be a major spontaneous metabolite of Q (Fig. 4A). Isorhamnetin levels increased gradually and achieved its maximal concentration at 12 hours of incubation. However, isorhamnetin was still unstable in medium alone. Its level decreased after 18 hours of incubation and might be remetabolized into another secondary metabolite (Fig. 4A). In the meantime, kaempferol and other unknown metabolites (RT = 7.6 minutes, 20.7 minutes, and 25 minutes) increased markedly as Q levels decreased, suggesting that kaempferol and other unknown metabolites (RT = 7.6 minutes, 20.7 minutes, and 25 minutes) are also spontaneous metabolites of Q (Fig. 4B). Unfortunately, some unknown metabolites (RT = 7.6 minutes, 20.7 minutes, and 25 minutes) could not be identified in this study. We hypothesized that these unknown metabolites (RT = 7.6 minutes, 20.7 minutes, and 25 minutes) were primary or secondary metabolites of Q—isorhamnetin or kaempferol.

Fig. 4C and D show changes in levels of Q and its metabolites in the extracellular fluid of peritoneal macrophages cultured with Q for different time periods. Similar to those in medium alone, Q and isorhamnetin showed a negative correlation as the incubation time was extended, suggesting that isorhamnetin is a major metabolite of Q in either the absence or the presence of peritoneal macrophages (Fig. 4C and D). Compared with the results from medium alone (Fig. 4A), isorhamnetin levels in the presence of macrophages increased quickly at 6 hours of incubation and achieved maximal concentration twice compared to that in medium alone, suggesting that macrophages enhance isorhamnetin production (Fig. 4C). Furthermore, isorhamnetin levels in both medium alone and macrophage extracellular fluids decreased to a similar low concentration at 24 hours of incubation, suggesting that nascent isorhamnetin in the extracellular fluid might subsequently enter the cells. In addition to isorhamnetin, kaempferol and other unknown metabolites (RT = 7.6 minutes, 20.7 minutes, and 25 minutes) increased markedly as the Q levels decreased, suggesting that kaempferol and other UQMs [RT = 20.7 minutes (UQM1), 25 minutes (UQM2), and 7.6 minutes (UQM3)] are also Q metabolites outside of macrophages (Fig. 4D). Compared with the results from medium

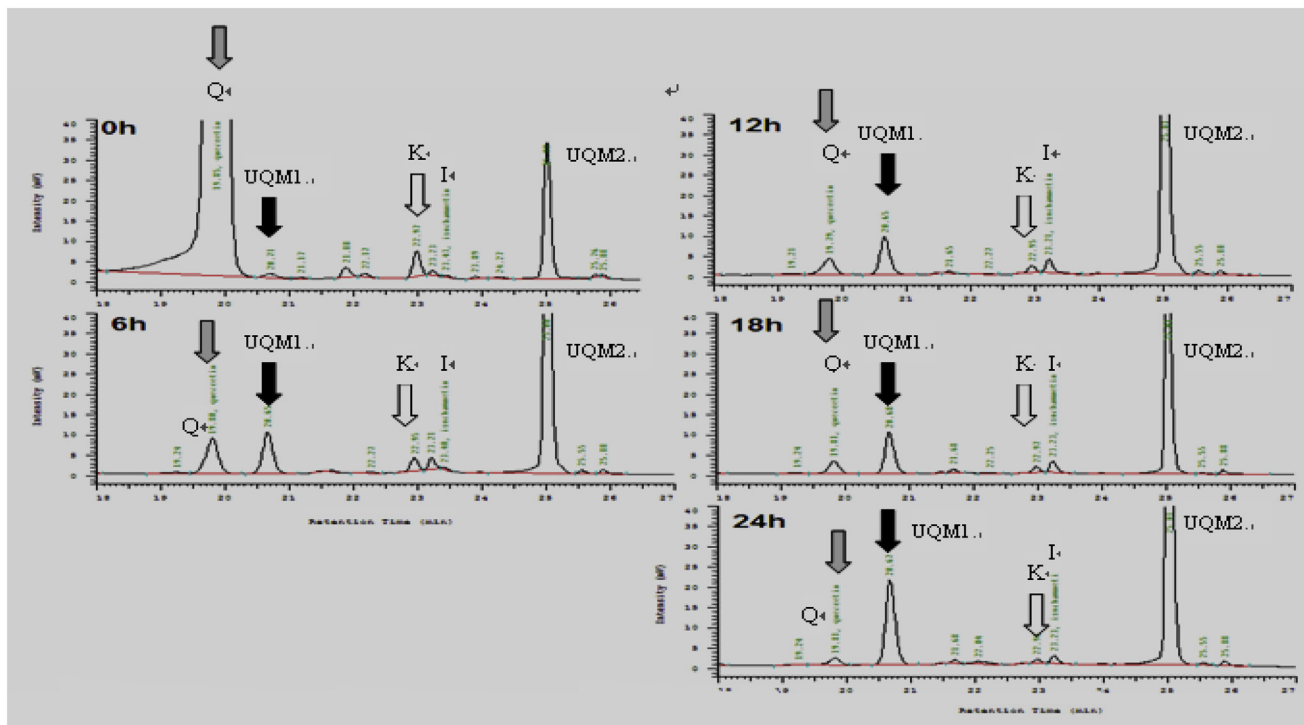


Fig. 2 – HPLC chromatograms showing quercetin metabolism in medium alone for different time periods. HPLC = high-performance liquid chromatography; I = isorhamnetin (3'-O-methyl quercetin); K = kaempferol; Q = quercetin; UQM = unknown quercetin metabolite.

alone (Fig. 4B), kaempferol levels increased quickly and decreased slowly as the incubation time was extended, suggesting that macrophages might enhance kaempferol production and a little amount of nascent kaempferol might enter the cells (Fig. 4D). Other changes in the levels of UQMs had the

same trend. Interestingly, an UQM (UQM4, RT = 9.9 minutes) just appeared in the macrophages, but not in medium-alone cultures. The nascent metabolites and remaining Q might subsequently enter the cells. Unfortunately, the unknown metabolites could not be identified in this study. However, our

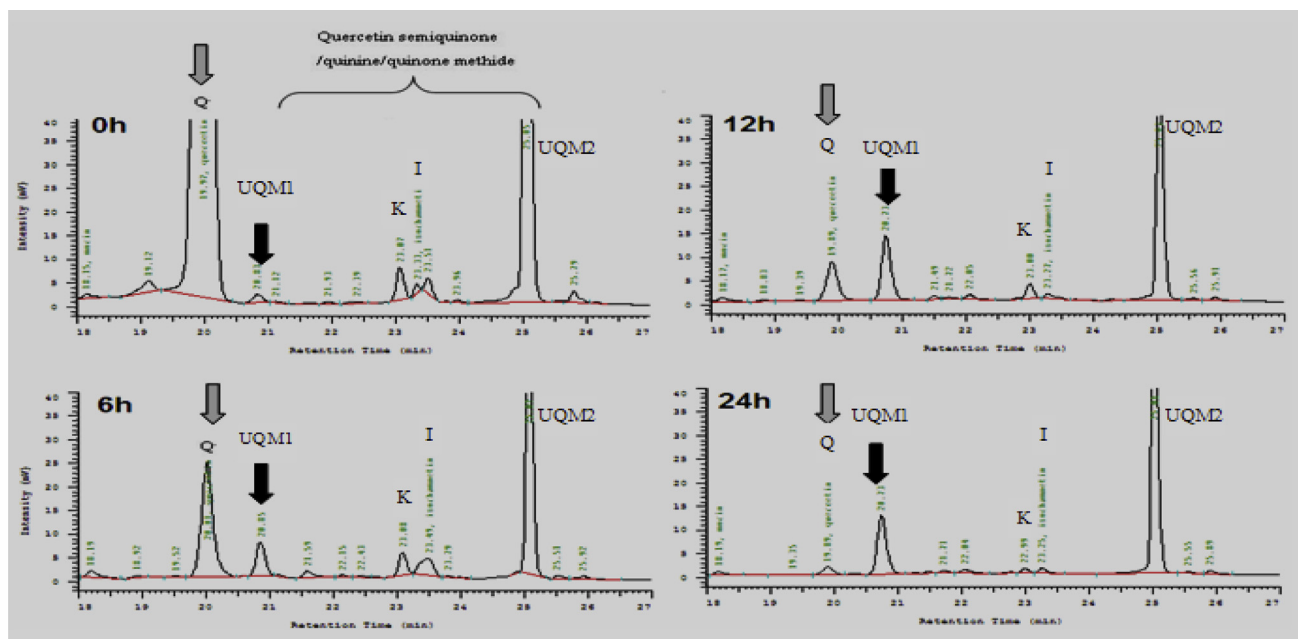


Fig. 3 – HPLC chromatograms showing extracellular fluid from peritoneal macrophages cultured with quercetin for different time periods. HPLC = high-performance liquid chromatography; I = isorhamnetin (3'-O-methyl quercetin); K = kaempferol; Q = quercetin; UQM = unknown quercetin metabolite.

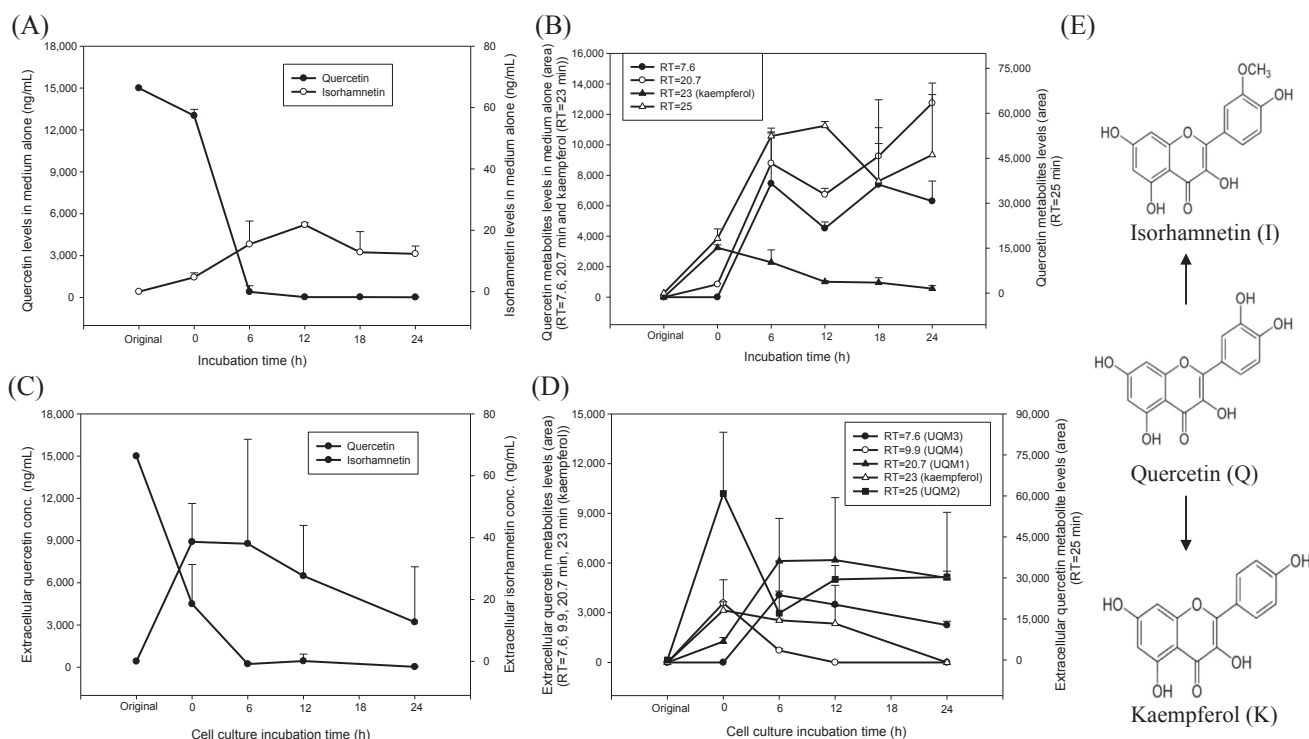


Fig. 4 – Changes in levels of quercetin and its metabolites in medium in the (A and B) absence or (C and D) presence of macrophages through different incubation times and (E) a proposed quercetin metabolic pathway. Values are presented as means \pm SEM ($n = 6$ biological determinations) according to HPLC chromatograms. The original cell density was 1×10^6 cells/mL. RT values were as follows: UQM1, 20.7 minutes; UQM2, 25 minutes; UQM3, 7.6 minutes; and UQM4, 9.9 minutes. HPLC = high-performance liquid chromatography; RT = retention time; SEM = standard error of the mean; UQM = unknown quercetin metabolite.

data provided evidence that Q might be directly metabolized into isorhamnetin or kaempferol in the absence or presence of macrophages (Fig. 4E).

3.3. Changes in levels of Q and its metabolites in intracellular fluids of peritoneal macrophages through different incubation times

Changes in the levels of Q and its metabolites in intracellular fluids of macrophages through different incubation times were measured using HPLC (Fig. 5). The levels of Q and its metabolites in intracellular fluids of macrophages were analyzed according to HPLC chromatograms (Fig. 6). The results showed that Q increased quickly and markedly in the intracellular fluid at 0 hour of incubation, indicating that most of Q was readily absorbed by macrophages. Furthermore, a trace amount of isorhamnetin (RT = 23.3 minutes) and an unknown metabolite (RT = 25 minutes, UQM2) that had been metabolized from Q outside the cells were found in the intracellular fluid at 0 hour of incubation, suggesting that isorhamnetin (RT = 23.3 minutes) and the unknown metabolite (RT = 25 minutes, UQM2) were also readily absorbed by macrophages. Interestingly, a novel unknown flavonoid compound (RT = 26.3 minutes) was also found in the macrophage intracellular fluid. The novel compound (RT = 26.3 minutes) was considered an endogenous Q metabolite (EQM) in the cells. As the incubation time was extended to 6 hours, Q

level decreased, whereas that of isorhamnetin increased, but levels of other metabolites (RT = 25 minutes and RT = 26.3 minutes) also decreased, indicating that isorhamnetin is a major metabolite of Q in macrophages. As the incubation time was extended to 12–24 hours, the levels of Q and all its metabolites decreased markedly, indicating that Q might be totally metabolized within 12–24 hours in macrophages (Fig. 6). Taken together, our results showed that Q, isorhamnetin, and UQM2 (RT = 25 minutes) might be absorbed by the macrophages (Fig. 6). The percentage of maximal uptake of Q was found to be 2.28% immediately after incubation; however, the uptake of Q persisted for about 24 hours (Fig. 7). It was found that quercetin glucosides can be transported by sodium-dependent glucose transporter SGLT1 across the apical membrane of enterocytes [10]. We presumed that Q, in its aglyconic form, could not enter immune cells through sodium-dependent glucose transporter, resulting in its low availability to immune cells or tissues [11]. Even though the percentage of maximal Q uptake by macrophages was just 2.28%, Q may have significant beneficial effects on immune cells. As Q entered the macrophages, it was further metabolized into isorhamnetin and an unknown EQM. Q itself has been found to inhibit endothelial nitric oxide synthase, microtubule polymerization, and mitotic progression in bovine aortic endothelial cells, suggesting that Q may have a potential role as a chemopreventive agent via suppressing endothelial cell proliferation and angiogenesis [12].

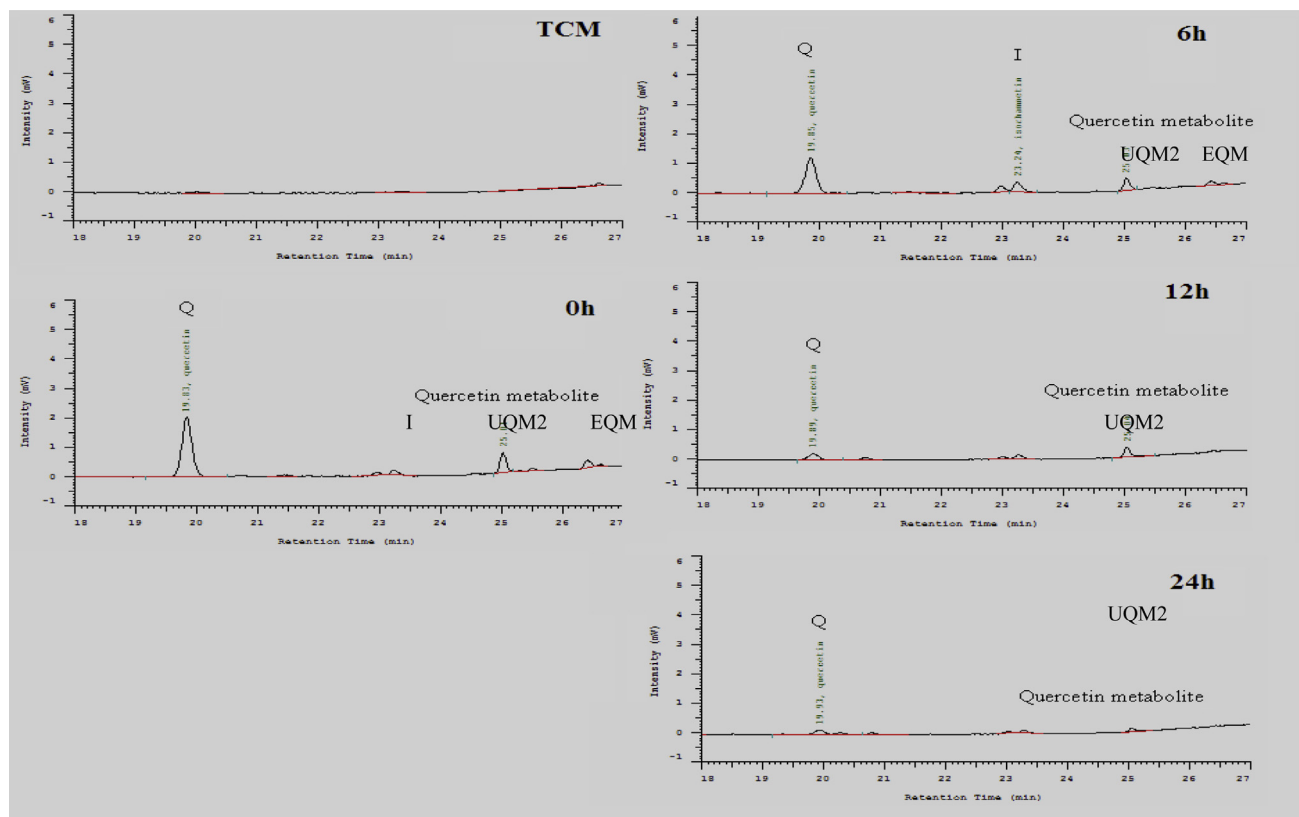


Fig. 5 – HPLC chromatograms showing intracellular fluid of peritoneal macrophages cultured with quercetin for different time periods. EQM = endogenous quercetin metabolite; HPLC = high-performance liquid chromatography; I = isorhamnetin (3'-O-methyl quercetin); K = kaempferol; Q = quercetin; TCM = tissue culture medium; UQMs = unknown quercetin metabolites.

Importantly, Q metabolites may further downregulate cyclooxygenase-2 transcription in human lymphocytes, exhibiting anti-inflammatory potential [13].

According to our data, we assumed that a catechol-O-methyltransferase was synthesized by macrophages to metabolize Q into isorhamnetin in both extra- and intracellular fluids [14]. It is reported that the extensive methylation of Q was found to limit its bioavailability in HepG2 cells [15]. Surco-Laos et al [16] also indicated a greater uptake of Q than its methylated derivatives by the nematode. Our results were identical to those of previous studies [15,16]. We also found that only a trace amount of isorhamnetin (3'-O-methyl quercetin) in extracellular fluid was absorbed by macrophages (Fig. 6A). In addition, flavonoids containing a catechol or dihydroxylated B-ring (e.g., Q) are easily oxidized. The oxidation may be carried out under physiological conditions or in medium alone in the presence of transition metals such as iron or copper. In the present study, we evidenced that Q might easily be metabolized into kaempferol due to spontaneous oxidation in the absence or presence of immune cells. Furthermore, certain enzymes such as tyrosinase or peroxidases may catalyze quinone formation. Q autoxidation in cells may also result in the production of reactive quinone and quinone methide species that are capable of reacting with cellular biomolecules and/or cellular thiols such as

glutathione and protein cysteine residues. Enzymatic oxidation of flavonoids may be beneficial to clear flavonoids from the cells via a reaction between quinone and glutathione, exporting the conjugate [17]. Uptake experiments using dermal fibroblasts indicated that exposure to Q resulted in the generation of two novel cellular metabolites, a 2'-glutathionyl Q conjugate and another product, putatively a quinone/quinone methide, suggesting that its formation is related to oxidative metabolism [18]. Based on our results, we hypothesized that tyrosinase, peroxidases, or quinone reductase might be produced by macrophages to metabolize Q outside or inside of cells into O-semiquinone (possibly UQM2), O-quinone (possibly UQM1) or quercetin quinoid (quinone methide) [17–20]. Most importantly, the present study exhibited that Q itself and some of its metabolites, including isorhamnetin and O-semiquinone (possibly UQM2), could enter macrophages, performing immunoregulatory functions in macrophages, subsequently producing some unidentified EQMs and isorhamnetin. Recently, Q was found to suppress lead-induced inflammatory response in rat kidneys through the reactive oxygen species-mediated mitogen-activated protein kinases and nuclear factor- κ B pathway [21]. Most recently, Q has been used in cancer prevention and therapy [22]. Unraveling Q uptake and metabolism using macrophages might further clarify Q immune effects. We have achieved some

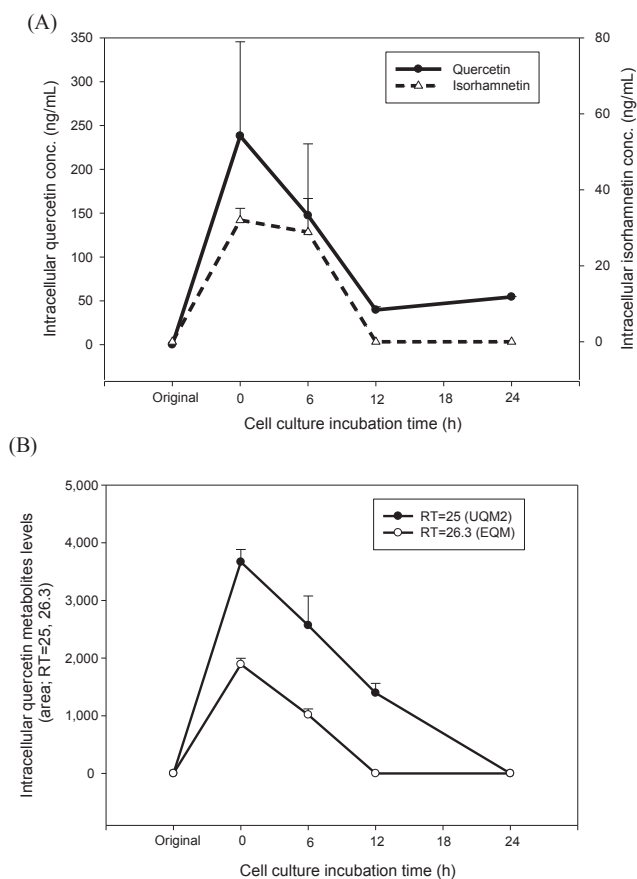


Fig. 6 – Changes in levels of quercetin and its metabolites in intracellular fluids of peritoneal macrophages cultured with quercetin through different incubation time. Values are presented as means ± SEM (n = 6 biological determinations) according to HPLC chromatograms. The original cell density was 1 × 10⁶ cells/mL. EQM = endogenous quercetin metabolite; HPLC = high-performance liquid chromatography; RT = retention time; SEM = standard error of the mean; UQM = unknown quercetin metabolites.

important results in the present study, although more data should be accumulated to further clarify Q cellular signaling in macrophages.

This study has some limitations. First, Q metabolites could be formed inside the macrophages and diffused into the extracellular medium. Our hypothesis that extracellular enzymes were involved might not be absolutely correct. Experiments with macrophage-conditioned medium should be performed to substantiate this hypothesis. Uptake and metabolic inhibitors should be performed in the macrophage uptake study to confirm Q biotransformation in immune cells.

4. Conclusion

Our results indicated that Q was unstable and easily oxidized in either the absence or the presence of macrophages. The remaining Q and its metabolites, including isorhamnetin and

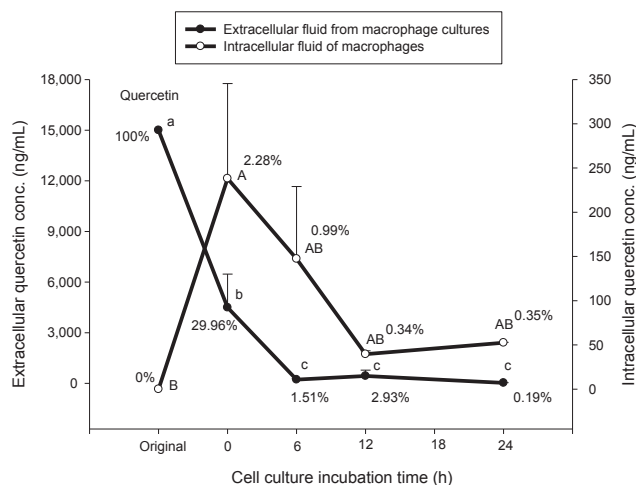


Fig. 7 – Changes in levels of quercetin in extra- and intracellular fluids of peritoneal macrophages. Values are presented as means ± SEM (n = 6 biological determinations) according to HPLC chromatograms. The original cell density was 1 × 10⁶ cells/mL. Points in the same plot without sharing a common letter are significantly different (p < 0.05) from each other assayed by one-way ANOVA, followed by Duncan's multiple range test. The relative percentage of quercetin content (%) = (residual content of quercetin at given incubation time/added original content of quercetin) × 100. In intracellular fluids, a relative percentage of quercetin content was calculated using the following equation: relative percentage of quercetin content (%) = [residual content of quercetin at given incubation time/(added original content of quercetin – residual content of quercetin in extracellular fluid at the given incubation time)] × 100. ANOVA = analysis of variance; HPLC = high-performance liquid chromatography; SEM = standard error of the mean.

an UQM [possibly Q⁻ (O-semiquinone)] might be absorbed by macrophages. The percentage of maximal Q uptake by macrophages was found to be 2.28% immediately after incubation; however, the uptake of Q might persist for about 24 hours. The uptake of Q by macrophages was greater than that of its methylated derivative isorhamnetin. As Q or its metabolites entered macrophages, those compounds were metabolized primarily into isorhamnetin, kaempferol, or EQMs.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This study was kindly supported by a research grant (NSC99-2628-B-005-004-MY3) from the National Science Council, Taipei, Taiwan, R.O.C.

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