Title

A novel mechanism for the promotion of quercetin glycoside absorption by megaloα-1,6-glucosaccharide in the rat small intestine

Running Title

α-1,6-glucosaccharide -enhanced absorption of flavonoid glycosides

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Abstract

The presence of α-1,6-glucosaccharide enhances absorption of water-soluble quercetin glycosides, a mixture of quercetin-3-*O*-β-D-glucoside (Q3G, 31.8%), mono (23.3%), di (20.3%) and more D-glucose adducts with α-1,4-linkage to D-glucose moiety of Q3G, in a ligated small intestinal loop of anesthetized rats. We enzymatically prepared α-1,6-glucosaccharides with different degrees of polymerization (DP) and separated them into a megaloisomaltosaccharide-containing fraction (M-IM, average DP=11.0) and an oligoisomaltosaccharide-containing fraction (O-IM, average DP=3.6). Luminal injection of either saccharide fractions promoted the absorption of total quercetin-derivatives from the small intestinal segment, and this effect was greater for M-IM than O-IM addition. M-IM also increased Q3G, but not quercetin aglycone, concentration in the water-phase of the luminal contents more strongly than O-IM. The enhancement of Q3G solubilization in the luminal contents may be responsible for the increases in the quercetin glucoside absorption promoted by α-1,6-glucosaccharides, especially M-IM. These results suggest that the ingestion of α-1,6-glucosaccharides promotes Q3G bioavailability.

Keywords: α-1,6-glucosaccharide; quercetin glycoside; absorption; rats

1. Introduction

Flavonoids, which contain diphenylpropanes ($C_6C_3C_6$), are secondary metabolites that are ubiquitously produced throughout the plant kingdom. Quercetin is a flavonoid that exists in various plant food products, including tea, wine, onions, and apples (Hertog, Hollman, & Katan, 1992). Quercetin is usually present in glycosylated forms, mainly as β-glucosides, in plant-based foods (Rechner, Wagner, Van Buren, Van De Put, Wiseman, & Rice-Evans, 2002; Crespy, Morand, Besson, Manach, Démigné, & Rémésy, 2001). The nature of the glycosylation most likely influences the absorptive efficiency of quercetin aglycone (Morand, Manach, Crespy, & Remesy, 2000). Quercetin glycosides have been suggested to function as anticarcinogenic, antidiabetic, and antiatherogenic agents (Bischoff, 2008; Murakami, Ashida, & Terao, 2008; Perez-Vizcaino, & Duarte, 2010). The beneficial effects of flavonoids depend on their bioavailability. Due to the low absorption rate of flavonoids in the intestine, enhancing bioavailability of flavonoid by other food ingredients is important for promotion of the beneficial effects. We have previously reported that non-digestible saccharides, such as fructooligosaccharide and difructose anhydride III (DFA III), promote the absorption of flavonoids (Matsukawa, Matsumoto, Chiji, & Hara, 2009). These non-digestible saccharides are known as “short-chain oligosaccharides”; the term “megalosaccharide” is used for saccharides with ten or more monosaccharide units, and the generic term of “oligosaccharide” is typically used for saccharides containing fewer than ten monosaccharide units (Thoma, Wright, & French, 1959).

Here, we prepared two groups of linear α-1,6-glucosaccharides, a megaloisomaltosaccharide-containing fraction (M-IM, average degree of polymerization (DP)

=11.0) and an oligoisomaltosaccharide-containing fraction (O-IM, average DP=3.6). We then investigated whether M-IM and O-IM promote the absorption of water-soluble quercetin glycosides in a ligated small intestinal segment in anesthetized rats in different ways. We used a mixture of one to seven D-glucose adducts of quercetin-3-*O*-glucoside (Q3Gn) as the water-soluble flavonoid glycoside.

2. Materials and methods

2.1. Chemicals

Linear α-1,6-glucosaccharide mixtures were prepared from maltodextrin by a bacterial glucosyl transferase (personal communication). We fractionated these mixtures to obtain M-IM, with an average DP=11.0, and O-IM, with an average DP=3.6. The quercetin glycoside mixture (Q3Gn) was kindly donated by San-Ei Gen F.F.I., Inc. (Osaka, Japan). Q3Gn mainly consisted of quercetin-3-*O*-β-D-glucoside (Q3G, 31.8%), mono (23.3%), di (20.3%) and tri to hepta D-glucose adducts with α-1,4-linkage to D-glucose moiety of Q3G. Quercetin and tamarixetin (monomethylquercetin) were obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), and purified each component of Q3Gn (Q3G and one to seven D-glucose adducts of Q3G) (San-Ei Gen F.F.I.) were used as standard compounds to quantify quercetin-derivatives by LC-MS. All other reagents and chemicals were of the highest commercially available grade.

2.2. Animals and diet

Male Wistar/ST rats weighing approximately 190 g (7 weeks old; Japan SLC,

Shizuoka, Japan) were housed in individual stainless-steel cages with wire-mesh bottoms. The cages were placed in a room with a controlled temperature (22 ± 2˚C), relative humidity (40-60%) and light (12 h-light/dark cycle at 8:00-20:00) for the duration of the study. The rats had free access to water and a flavonoid-free diet based on the AIN93G formulation (Reeves, Nielsen, & Fahey, 1993) and were allowed to acclimate to these conditions for at least 7 d. This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

2.3. Experimental procedure using small intestinal loops

After overnight food deprivation, the rats were anesthetized with an intraperitoneal administration of ketamine (80 mg/kg body weight; Ketaral, Daiichi Sankyo, Tokyo, Japan) containing xylazine (12 mg/kg body weight; MP Biomedicals, Irvine, CA, USA), and an abdominal midline incision was made. The small tip (7 – 8 mm) of a polyethylene catheter (SP 28; ID 0.4 mm, OD 0.8 mm; Natsume Seisakusyo, Tokyo, Japan) connected to a silicone catheter (Silascon no. 00, ID 0.5 mm, OD 1.0 mm; Dow Corning, Kanagawa, Japan) was inserted into the portal vein (Hara, Yamada, & Kiriyama, 1991). A ligated small intestinal loop (15 cm in length) was prepared in the jejunum in each rat. The jejunal segments were washed with saline delivered through two small punctures, and the intestine was ligated between 5 and 20 cm distal to the ligament of Treitz. A test solution (1.5 mL) containing 10 mmol/L Q3Gn in MOPS buffer (pH 6.5) with or without 20 mmol/L M-IM or O-IM was then injected into the segment. The isotonicity of the solutions was adjusted with NaCl. Portal blood was collected through the portal catheter at 7.5, 15 and 30 min after Q3Gn injection.

The luminal solution was collected at 30 min after injection for measurements of the remaining quercetin-derivatives. During the experiment, additional ketamine/xylazine was injected to ensure that the rats remained anesthetized, and body temperature was maintained with heating pads.

2.4. Plasma and luminal solutions treatments for LC-MS analyses

Plasma (100 μL) from the portal vein was acidified (to pH 4.9) with 10 μL 0.58 mmol/L acetic acid and treated with (total flavonoids) or without (unconjugated flavonoids) 10 μL of β-glucuronidase (*Helix pomatia* extract, Sigma G0876, 9,600 U/mL of β-glucuronidase and 390 U/mL of sulfatase) for 30 min at 37˚C. To extract quercetin-derivatives, the reaction mixture was added to 100 μL of methanol, heated at 100˚C for 1 min, and centrifuged to collect the supernatant. The procedure was repeated three times. The collected supernatant was applied to a C18 cartridge (Oasis HLB, Waters Co. Ltd., Milford, MA, USA). After washing with 1 mL water, the quercetin-derivatives were eluted with methanol, dried and dissolved in 100 μL of 50% methanol (sample solution). The luminal solution was prepared in the same manner.

2.5. Solubilization of quercetin glucoside in α-1,6-glucosaccharide solution with in vitro study

Q3G was suspended at a concentration of 10 mmol/L in the deionized water with or without 20 mmol/L O-IM or M-IM. These mixtures were incubated at 37˚C for 30 min. After centrifugation, Q3G in the supernatant were analysed by LC/MS.

2.6. LC-MS analysis

Quercetin and its metabolites were identified and quantified by liquid chromatography mass spectrometry (LC-MS) using an electric spray ionization (ESI) interface (TSQ Quantum Access with Accela High Speed LC System, Thermo Fisher Scientific Inc., MA, USA). The temperature of the capillary heater and the vaporization heater were maintained at 220˚C and 450˚C, respectively. LC/ESI-MS was carried out in scan mode from (*m*/*z*) to + 3,000 and in selected ion monitoring mode (*m*/*z*) +273 for naringenin (internal standard), (*m*/*z*) +303 for quercetin, (*m*/*z*) +317 for monomethylquercetin (isorhamnetin and tamarixetin), and (*m*/*z*) +465, 627, 789, 951, 1113, 1275, 1437, 1599 for Q3G1 to Q3G8, respectively. The LC system was fitted with a 1.7 μm C18 column (Acquity UPLC BEH, 2.1 × 100 mm, Waters Co. Ltd., Milford, MA, USA) set at 40˚C. Solvent A (water/methanol/formic acid, 70:30:0.1) and solvent B (methanol/ formic acid, 100:0.1) were prepared. The flow rate of the mobile phase was 0.2 mL/min, and the proportion of solvent B was raised linearly from 20% to 80% over 8 min, then reduced linearly back to 20% over 1 min and subsequently maintained at the initial condition for 1 min.

2.7. Calculation and statistics

The concentrations of internal standards and quercetin-derivatives were calculated from the peak area of each mass spectrum and calibration curves of standard compounds. The concentrations of conjugated quercetin-derivatives in the plasma were extrapolated from the quercetin aglycone or monomethylquercetin concentrations after enzymatic treatment. All values are expressed as the means and standard error of the mean. Statistical analysis was performed with one-way or two-way ANOVA, and the differences among treatment groups were determined using Tukey-Kramer's test. A difference of $P < 0.05$ was considered

significant.

3. Results

No quercetin-derivatives were detected in the blood or luminal solution in rats before Q3Gn injection. We found quercetin aglycone, methylquercetin and conjugated forms of quercetin in the portal blood after Q3Gn injection. Quercetin aglycone was the most prevalent Q3Gn metabolite detected. A two-way ANOVA revealed that the plasma concentrations of quercetin aglycone (Fig. 1A), monomethylquercetin (Fig. 1B), glucuronide- and sulfate-conjugated derivatives (Fig. 1C) and total quercetin-derivatives (Fig. 1D) in portal blood were significantly influenced by saccharide and time. At 15 min after the injection of the test solution, the blood concentrations of quercetin aglycone and monomethylquercetin were significantly higher in M-IM group, but not in the O-IM group, compared with the control group ($P < 0.05$). The plasma concentrations of glucuronide- and sulfate-conjugates were greater in the M-IM group than in the O-IM group at 15 min after Q3Gn injection ($P < 0.05$). At 7.5 and 15 min after injection of the test solution, the total concentration of quercetin-derivatives was significantly higher in the M-IM group than in the control group ($P < 0.05$). In the portal blood, the Q3G concentration was very low, and there was no difference between M-IM and O-IM. Neither Q3G2 nor Q3G3 were detected (data not shown).

The water-soluble fraction of the luminal contents at 30 min after injection of the test solution contained mainly Q3G, which is a partly digested product of Q3Gn. The levels of aglycone and other glycosides were very low. Conjugated forms of quercetin metabolites were not detected. The supplementation of Q3Gn with M-IM or O-IM dramatically increased the concentrations of the soluble quercetin-derivatives in the luminal solution (Fig. 2, $P <$

0.05). The soluble quercetin-derivatives in the intestinal lumen were significantly higher in the M-IM group than in the O-IM group.

In an *in vitro* study, solubilized Q3G were much higher in the M-IM solution than in deionized water (control) and also than O-IM solution (Fig. 3). Especially, solubilized Q3G in the M-IM solution was more than 2.5 times higher compared with that in deionized water.

4. Discussion

This study demonstrates that M-IM (DP=11.0) and O-IM (DP=3.6) enhanced the absorption of Q3Gn in the small intestinal loop, and the absorption was more prominently enhanced by M-IM. These enhancements were accompanied by the solubilization of Q3G in the luminal contents by M-IM.

Several different pathways are involved in flavonoid absorption in the intestine. The Q3Gn used in the present study is one to seven D-glucose adducts of Q3G, which is readily hydrolyzed to Q3G by α-glucosidases in the intestinal lumen. There is evidence that Q3G is absorbed from the intestines via an intracellular pathway. In a previous study, it was also evident that a non-digestive oligosaccharide, DFA III, promotes the intestinal absorption of αG-rutin, another water-soluble flavonoid glycoside, via the paracellular absorption through the tight junction (Matsumoto, Matsukawa, Chiji, & Hara, 2007). These findings suggested that Q3Gn was absorbed from the intestine via both intracellular and paracellular pathways. In the paracellular pathway, flavonoid glycosides are usually absorbed as glycosides without methylation or conjugation. In contrast, during absorption via the intracellular pathway, most flavonoid glycosides are deglycosylated by enterocyte β-glucosidases, a cytosolic β-glucosidase with broad specificity (Németh et al., 2003). In the present study, we found no

Q3Gn in the portal blood and detected considerable amounts of conjugated quercetin forms. Linear α-1,6-glucosaccharides, especially M-IM, increased the portal concentration of the conjugated quercetin. These results indicate that M-IM supplementation promotes the intracellular absorption of quercetin-derivatives. Moreover in the present study, we found that the M-IM increased the concentrations of total quercetin-derivatives in the portal blood at 15 min. We calculated the approximate absorption rate of quercetin-derivatives into the portal vein at 15 min using the value of portal blood flow (Vorobioff, Bredfeldt, & Groszmann, 1984), and the estimated percentages of absorbed quercetin glucosides in the portal blood were 14.6 ±2.0%, 23.4 ± 3.9%, 32.5 ± 7.1% for control, O-IM, M-IM groups, respectively.

In the intestinal lumen, Q3G derived from Q3Gn is further hydrolyzed to quercetin aglycone by lactase-phlorizin hydrolase (LPH) in the brush border membrane and/or intracellular β-glucosidase in the small intestinal epithelium (Day et al., 2000a; 1998). The aglycone generated in the intestine is immediately metabolized by phase-II enzymes located in intestinal epithelial cells (Day, Bao, Morgan, & Williamson, 2000b). Quercetin aglycone is converted into conjugated compounds, such as glucuronide and sulfate, by uridine 5'-diphosphate glucuronosyltransferase (UGT) and phenol sulfotransferase (PST), respectively. In this study, we found that the plasma concentration of quercetin aglycone and monomethylquercetin readily increased after the injection of Q3Gn concomitant with M-IM or O-IM. One possible reason for the large increase in aglycone is that the inflow of quercetin exceeds the capacity of UGT and PST conjugation in the intestinal epithelial cells. The increases in the glucuronide-conjugated or sulfate-conjugated quercetin-derivatives in the portal blood were slower than that of quercetin aglycone after M-IM injections. These results also support the hypothesis that M-IM supplementation promoted the intracellular absorption

of Q3Gn.

We demonstrate a prominent increase in Q3G concentration in the water-phase fraction of the luminal contents after supplementation with M-IM or O-IM. Quercetin aglycone in the water-phase fraction was very low and not changed by supplementation of α-1,6-glucosaccharides. We speculate that aglycone produced from Q3G by LPH is readily absorbed into the epithelial cells, which is probably responsible for increase in Q3Gn absorption by α-1,6-glucosaccharides. We estimated the solubilization of Q3G by α-1,6-glucosaccharides from the concentration of Q3G in the water-phase of the remaining luminal contents at 30 min, and the estimated percentages were 2.6 ±0.3%, 9.7 ± 1.7%, 13.1 ± 2.3% for control, O-IM, M-IM groups, respectively. In an *in vitro* study, we confirmed that the solubilization of Q3G dramatically increased by supplementation of M-IM. This is the direct evidence that M-IM solubilized Q3G and the higher solubility in the intestinal fluid including M-IM is responsible for the enhancement of the flavonoid absorption in M-IM group. Interestingly, the enhancement in the absorption rate of quercetin-derivatives is stimulated to a greater degree by supplementation with M-IM than by supplementation with O-IM. The solubilization of Q3G in the intestinal lumen is a novel physiological function for α-1,6-glucosaccharides and has not been demonstrated previously for other sugars.

In conclusion, M-IM promotes the absorption rate of Q3Gn in the small intestine and may enhance the bioavailability of Q3Gn. The modulation of the solubility of Q3G derived from Q3Gn by M-IM in the intestinal lumen may be one determinant of the absorption of quercetin glycoside and may promote the beneficial effects of quercetin glycosides.

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Figure Captions

Fig. 1. Plasma concentrations of quercetin aglycone (A), monomethylquercetin (B), conjugated (glucuronide or sulfate) quercetin-derivatives (C) and total quercetin-derivatives (D) in the portal vein after injection of 10 mmol/L Q3Gn mixture in a rat intestinal loop without glucosaccharide (control: square) or with 20 mmol/L O-IM: circle (average DP=3.6) or M-IM: triangle (average DP=11.0). Each value is expressed as the means ± SEM (n = 5–6). Two-way ANOVA *P* values were (A) 0.014, <0.001, and 0.49; (B) 0.001, <0.001, and 0.68; (C) 0.002, <0.001, and 0.86; (D) 0.001, <0.001, and 0.92 for saccharides, time, and saccharides × time, respectively.[a,b] Means not sharing a common letter differ significantly (Tukey-Kramer's test, $P < 0.05$).

Fig. 2. Concentrations of quercetin and Q3Gn in the water-phase fraction of the remaining luminal contents in the rat intestinal loop after injection of 10 mmol/L Q3Gn mixture in a rat intestinal loop without glucosaccharide (control) or with 20 mmol/L O-IM (average DP=3.6) or M-IM (average DP=11.0). Each value is expressed as the means ± SEM (n = 5–6). One-way ANOVA *P* values were < 0.001.[a,b] Means not sharing a common letter differ significantly (Tukey-Kramer's test, $P < 0.05$).

Fig. 3. Solubilization of Q3G in the water-phase of 10 mmol/L Q3G without glucosaccharide (control) or with 20 mmol/L O-IM (average DP=3.6) or M-IM (average DP=11.0). Each value is expressed as the average of 3 observations.

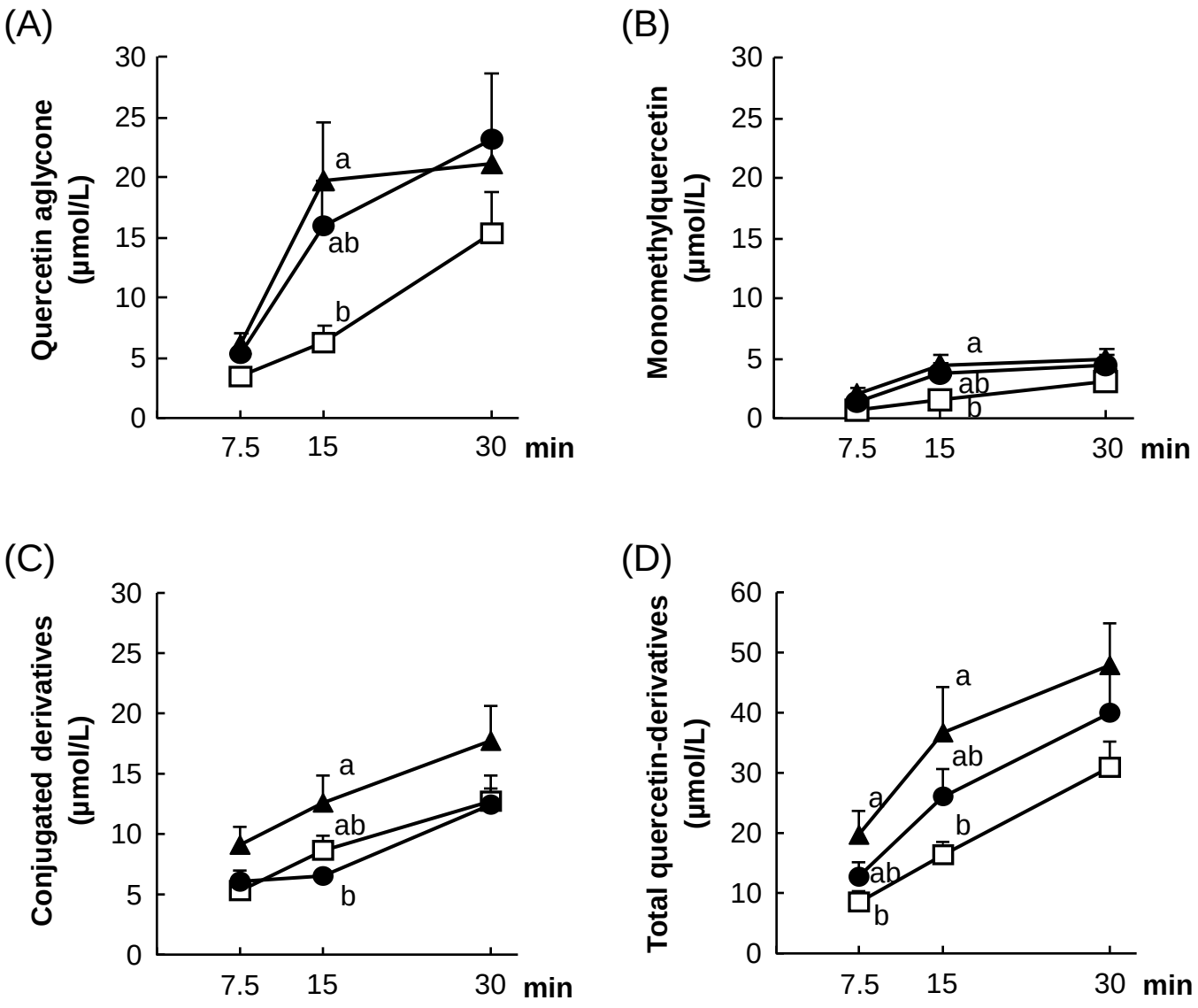
(A)
Quercetin aglycone (µmol/L)
30
25
20
15
10
5
0
a
ab
b
7.5
15
30
min
(B)
Monomethylquercetin (µmol/L)
30
25
20
15
10
5
0
a
ab
b
7.5
15
30
min
(C)
Conjugated derivatives (µmol/L)
30
25
20
15
10
5
0
a
ab
b
7.5
15
30
min
(D)
Total quercetin-derivatives (µmol/L)
60
50
40
30
20
10
0
a
ab
b
a
ab
b
7.5
15
30
min

Fig. 1

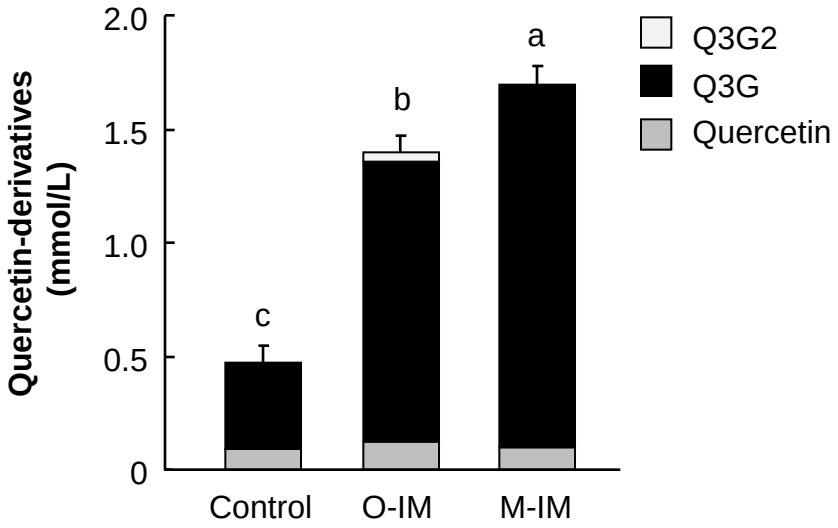
Quercetin-derivatives (mmol/L)
2.0
1.5
1.0
0.5
0
c
b
a
Control
O-IM
M-IM
Q3G2
Q3G
Quercetin

Fig. 2

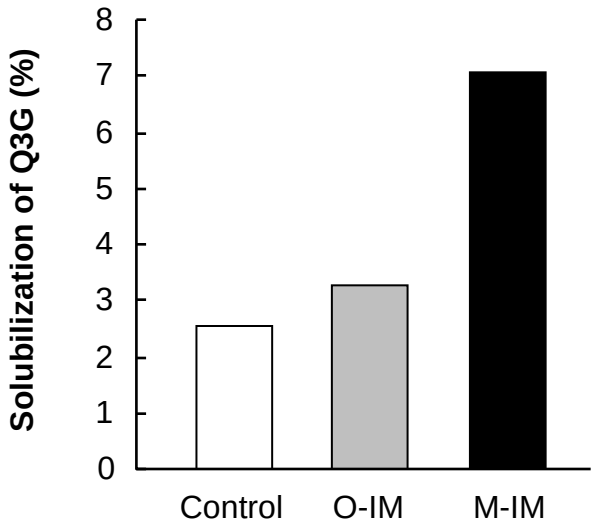

Fig. 3