



## Assessment of intestinal permeability of EGCG by piperine using Caco-2 cell monolayer system

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**Abstract** (–)-epigallocatechin-3-gallate (EGCG), a flavonoid found in green tea, is known to have low bioavailability. In this study, we determine whether piperine, a natural bioenhancer, can increase the absorption rate of EGCG. Using a Caco-2 cell monolayer, permeability experiments were performed in Hanks' balanced salt solution (HBSS) and EGCG stability was adjusted to pH 6.5 and pH 5.5 by ascorbic acid treatment. When HBSS was adjusted to pH 6.5, EGCG remained at 94.78% for up to 2 h and remained at 86.04% after 4 h and the net efflux decreased compared to the control. As a result, uptake was significantly increased in the piperine co-administered group compared to the EGCG-alone group, showing that piperine increased the permeability of EGCG in the Caco-2 cell monolayer. These results suggest that piperine inhibits EGCG glucuronidation and efflux, allowing for greater absorption of EGCG.

**Keywords** Ascorbic acid · Caco-2 cell · Efflux · Monolayer

### Introduction

The use of natural products has a long history, and the development of natural supplements is ongoing due to their fewer side effects and easier access compared with pharmaceuticals. *In vitro* studies have shown significant results for natural products whereas the *in vivo* bioavailability and lipid solubility for these have not been always shown to be similar ones.

The major problem regarding bioavailability is the inability of these products to cross the lipid membranes of the intestine. The bioavailability can be improved with the use of novel delivery systems, such as liposomes, marinosomes, niosomes, and lipid-based systems that can enhance the rate of release as well as the capacity to cross lipid-rich biomembranes [1].

A “bioenhancer” is an agent capable of enhancing the bioavailability and bioefficacy of a particular drug, without any typical pharmacological activity of its own at the dose used [2].

The main component of green tea extract, catechin, contains the phenolic compound (–)-epigallocatechin gallate (EGCG) [3]. Compared to other beverages, green tea contains high levels of nitrogen, polyphenols, sugars, organic acids, vitamins, and minerals. Green tea polyphenols are mostly flavanols. The major green tea catechins are (+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), (–)-gallocatechin gallate (GCG), (+)-gallocatechin (GC), and other alkaloids containing caffeine, theobromine, and theophylline [4].

The flavonoids extracted from green tea have very low bioavailability. One pharmacokinetic study showed that, after oral administration of green tea to rats, only 14% of EGC, 31% of EC, and <1% of EGCG appeared in the blood [5]. For humans, after administration of 3 g of decaffeinated green tea, the maximum plasma concentrations for EGCG, EGC, and EC were 0.57, 1.60, and 0.6 M, respectively [6]. The apparent permeability coefficient (P<sub>app</sub>) values of EGCG and EGCG niosomes were found to be 0.88±0.09 and 1.42±0.24 cm/sec in one *in vitro* study [3]. In another study, EGCG nanoformulations containing EGCG and

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piperine polymers in zein nanocarriers showed higher anticancer activity on various cell lines (HL60, SCC40, MCF7, HeLa, and Colo205) than pure EGCG [7]. The coadministration of piperine in CF-1 mice in another study improved the EGCG uptake in the small intestine by approximately 1.7-fold compared to the EGCG-treated group and increased by around 1.3-fold in plasma [8].

Piperine is a major component of the long pepper (*Piper longum* Linn) and the black pepper (*Piper nigrum* Linn). Both spices are commonly found in Asian diets [9]. Piperine can inhibit metabolism in the gut by inhibiting the enzyme CYP3A in epithelial cells, thereby enhancing bioavailability [10]. In addition, P-glycoprotein (P-gp) inhibition enhances bioavailability by inhibiting efflux during intestinal absorption [11]. For this reason, piperine may also enhance the intestinal absorption of EGCG.

Caco-2 cell monolayers are a useful experimental tool for evaluating the *in vitro* permeability of new compounds and may play an important role in the study of EGCG gastrointestinal permeability. In this study, we performed permeability experiments by creating experimental conditions to maintain the stability of EGCG in a Caco-2 cell monolayer. For EGCG stability evaluation, EGCG content was measured by HPLC in Hanks' balanced salt solution (HBSS) treated with bovine serum albumin (BSA), human serum albumin (HSA), hydrogen chloride, or ascorbic acid. We evaluated whether the permeability of EGCG is enhanced by piperine while maintaining a HBSS pH of 7.4–6.5, which is required for permeability evaluation in Caco-2 cell monolayers.

## Materials and Methods

### Materials

In this experiment, EGCG (>98%) was purchased from Tokyo Chemical Industry, Japan, piperine (>97%) from was purchased from WAKO, and verapamil and ascorbic acid were purchased from Sigma. For cell culture, fetal bovine serum (FBS) and a 0.22- $\mu$ m bottle top filter system were purchased from Corning. Dulbecco's modified Eagle medium (DMEM), sodium bicarbonate solution, penicillin-streptomycin, and non-essential amino acid (NEAA) were purchased from Gibco. Cell proliferation kits (XTT, Welgene) were purchased for cytotoxicity experiments. Cell monolayer formation and transport experiments were performed using Corning<sup>®</sup> 24 mm Transwell-COL collagen-coated 0.4- $\mu$ m pore polytetrafluoroethylene (PTFE) membrane inserts and HBSS, Gibco). For HPLC analysis, acetonitrile (ACN) and methyl alcohol were purchased from Daejung at HPLC grade.

### Stability of EGCG on HBSS

In order to measure the stability of EGCG depending on the pH condition, HBSS was adjusted to pH 3.5, 4.5, 5.5, 6.5, and 7.5 using 0.1 N hydrogen chloride. EGCG (500  $\mu$ M) was prepared in HBSS to make a 10% HBSS solution to the final 50  $\mu$ M EGCG while being incubated in a shaking incubator at 37 °C. 100- $\mu$ L

aliquots of the above solution were taken at 0, 1, 2, 3, and 4 h. In order to examine EGCG stability by BSA and HSA treatment, 1 mM EGCG was prepared in HBSS and the concentrations of BSA and HSA used as stabilizers were prepared at 1.5 mM. 250  $\mu$ L of EGCG and stabilizer (BSA and HSA) were mixed to make a mixed reagent and was left to stand for 2 min. EGCG and PBS were used as the control. A 10% HBSS solution was prepared using the reagents and then the experiment was conducted in the same manner as above. EGCG stability by ascorbic acid treatment was adjusted to pH 6.5 and pH 5.5 by adding 2.14 mM and 3.75 mM of acid, respectively, to HBSS. 50 of  $\mu$ M EGCG was added to HBSS containing ascorbic acid and then reacted for 4 h by the same method as above, then the aliquots were analyzed by HPLC.

### Cell culture

We purchased Caco-2 cells from Korea Cell Bank (Seoul, Korea) and added DMEM, 10% FBS, 1% NEAA and 1% penicillin-streptomycin. We adjusted the solution to pH 7.0–7.3 with HCl or NaOH and filtered it under reduced pressure using a 1 L bottle top filter system (0.22  $\mu$ m). For cell passage culture, we used medium containing NEAA. In the Caco-2 cell monolayer experiment, all compositions were the same, but we did not use NEAA. We placed cells in a CO<sub>2</sub> incubator at 37 °C with 90% relative humidity and 5% CO<sub>2</sub>. We replaced the culture medium 2–3 times per week. After growing up to 80–90% confluency, we detached the cells from the culture flask by adding a 0.25% trypsin-EDTA solution to seed in plates or flasks. In this experiment, we used cell passage numbers 19–28.

### Caco-2 cell monolayers

Caco-2 cells were cultured as described above. We seeded Caco-2 cells in 6-well plates with 0.4  $\mu$ m pore size collagen-coated inserts and PTFE membranes (Corning, USA) to form Caco-2 cell monolayers. We placed 3 mL of DMEM medium on the basolateral side of the well, and dispensed 2 mL of cell solution containing  $1 \times 10^5$  cells on the apical side. We incubated the 6-well plate containing cells at 37 °C and 5% CO<sub>2</sub>. After incubation for 48–72 h, we observed the cells by light microscopy, discarding the old media if the cells were attached, and replacing it with new media (3 mL on the basolateral side and 2 mL on the apical side). We performed media exchange of transwell cells every 3 days in the first week and every 2 days thereafter for a total of 21 days. Before carrying out the transport experiment, transepithelial electrical resistance measurements (Millicell ERS-2 Voltohmmeter) were taken to confirm the formation of Caco-2 cell monolayers. Before measurement, the probe was sterilized with 70% ethyl alcohol and soaked in DMEM. The 6-well plate to be measured in the incubator was brought to the clean bench, and the probe was placed on the apical and basolateral sides of the transwell. Only those with a value of 230  $\Omega$ /cm<sup>2</sup> or more were used. If the measured epithelial electrical resistance was less than 230  $\Omega$ /cm<sup>2</sup>, tight junctions formed as differentiated Caco-2 cells were not

formed properly and these were excluded from transport experiments.

### Cytotoxicity

Caco-2 cells were diluted with DMEM to  $1.5 \times 10^5$  cells/well and 200  $\mu\text{L}$  of this dilution was then dispensed into 96-well plates. The plates were incubated in a 5%  $\text{CO}_2$  incubator at 37 °C for 24 h. For cytotoxic samples, the sample with the highest ratio of EGCG and piperine (1:2) and the sample mixed with EGCG and verapamil (1:0.5) were dispensed by concentration on a 96-well plate, which was then incubated for 4 h at 37 °C in a 5%  $\text{CO}_2$  incubator. After washing three times, 40  $\mu\text{L}$  of XTT solution was added and the absorbance was measured at 450 and 690 nm using an ELISA reader. The cell survival rate was obtained by selecting the cells which were treated with the test sample for 4 h, mixing 20  $\mu\text{L}$  of this suspension with 20  $\mu\text{L}$  of trypan blue, and then dispensing on a counting slide to determine the cell count (NanoEnTek).

### Cellular transport studies

The EGCG permeability and prediction of absorption in Caco-2 monolayers followed the procedure described previously [12] with some minor modifications. Six-well plates in which a Caco-2 monolayer was incubated for 21 days were taken out of the incubator, the medium was removed, HBSS was used as a transport medium, and the plates were incubated for 1 h. HBSS was used after adding 10 mM ascorbic acid drop-by-drop and adjusting HBSS to pH 6.5. After 1 h, the HBSS was removed and 1.5 mL of each drug was added to the apical part of the plate to observe the experiment on the basolateral side from the apical side. After adding 2.5 mL of fresh HBSS to the basolateral side, 1 mL of the basolateral side was collected at 0.5, 1, 2, 3, and 4 h for analysis. To observe the transfer from the basolateral side to the apical side, the existing HBSS was removed, and 1.5 mL of HBSS without sample was added to the apical side of the plate and 2.5 mL of HBSS containing each sample was added to the basolateral side. During incubation at 37 °C, 1 mL of sample was collected from the apical side at 0.5, 1, 2, 3, and 4 h and filtered through a syringe filter (13 mm, 0.45  $\mu\text{m}$ , PVDF, Advantec) and then used for HPLC analysis. The Papp of EGCG was calculated using the following equation:

$$P_{app} = dQ/dt \times \frac{1}{A \times C_0} \quad (1)$$

where  $dQ/dt$  is the permeability rate ( $\mu\text{g}/\text{sec}$ ),  $C_0$  is the initial concentration in the donor chamber ( $\mu\text{g}/\text{mL}$ ), and  $A$  is the surface area of the filter (4.67  $\text{cm}^2$  in this study). After the transfer experiment, the 6-well plate with the Caco-2 monolayer was rinsed with HBSS, both apical and basal sides, 0.1 mg/mL lucifer yellow solution was added (1.5 mL) into the apical compartment, and HBSS buffer was added to the basal compartment. Plates were wrapped in foil and incubated for 1 h in a 37 °C incubator

while blocking light. Then 200  $\mu\text{L}$  was sampled from the basal compartment and measured using a 485 nm excitation filter and an emission filter of 527 nm on a fluorometer. The percentage of permeability was calculated from the fluorescence values as follows:

$$\% \text{ permeability} = \frac{\text{samples-blank}}{\text{Luciferyellow-blank}} \times 100 \quad (2)$$

A permeability of <3% is acceptable.

### Analysis by HPLC

The analysis of EGCG using HPLC followed a method previously described with some minor modifications [3,13,14]. A Shiseido Nanospace SI-2 series HPLC system was used with two solvent pumps and a UV detector. For chromatography, a Shiseido Capcell Pak C 18 UG 120 (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm) was used and the column temperature was 40 °C. The mobile phase used 0.1% acetic acid in distilled water and 0.1% acetic acid in acetonitrile. All samples were analyzed at a UV wavelength of 280 nm, flow rate was 1 mL/min, injection volume was 35  $\mu\text{L}$ , and the autosampler temperature was maintained at 4 °C.

### Data analysis

We performed all experiments at least three times. Data is expressed as the mean and the standard deviation ( $\pm$ SD). We evaluated the significance of differences with Student's paired  $t$ -test in SPSS 21.0, setting the criterion of significance at  $p < 0.05$ .

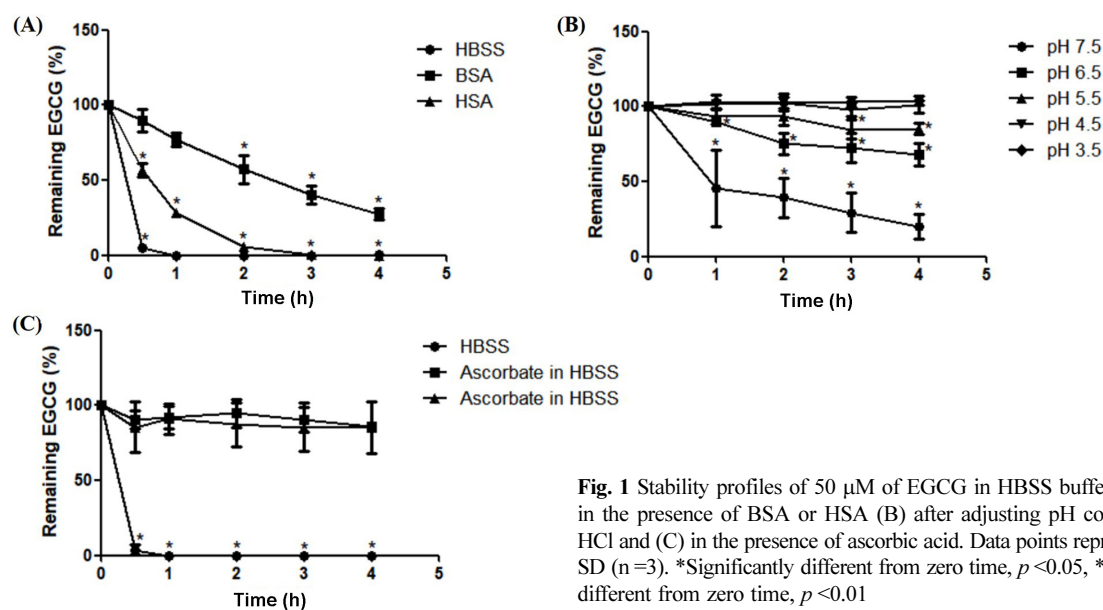
## Results and Discussion

### EGCG stability on cellular assay

In order to examine the stability of EGCG *in vitro*, the stability of EGCG on HBSS was measured by treatment with BSA and HSA, which are known to increase stability by protein binding. EGCG content was barely detected after 4 h in HBSS in the permeability assay. In contrast, the BSA-treated group showed increased EGCG stability. The BSA-treated group remained at around 57.19% EGCG after 2 h. The HSA-treated group showed higher stability than the control group; however, EGCG stability was more effective in BSA than HSA (Fig. 1A). The level of protein binding was higher than 97% for both HSA and BSA.

The stability of EGCG in the weakly acidic and neutral range of HBSS buffer was surprisingly reduced. EGCG decreased to around 80% after 4 h in HBSS at pH 7.5 and remained at around 100% after 4 h in HBSS at pH 3.5 (Fig. 1B). EGCG was found to be very unstable under pH neutral conditions. After 4 h, EGCG stability under pH 5.5, 4.5, and 3.5 conditions remained at 80, 100, and 100%, respectively, but permeability experiments were difficult *in vitro* due to low pH values.

In consideration of the decrease of stability by redox reaction in neutral conditions, EGCG was treated with ascorbic acid to



**Fig. 1** Stability profiles of 50  $\mu\text{M}$  of EGCG in HBSS buffer (37  $^{\circ}\text{C}$ ) (A) in the presence of BSA or HSA (B) after adjusting pH condition using HCl and (C) in the presence of ascorbic acid. Data points represent mean  $\pm$  SD ( $n=3$ ). \*Significantly different from zero time,  $p<0.05$ , \*\*significantly different from zero time,  $p<0.01$

evaluate its stability in weak alkaline conditions. In HBSS adjusted to pH 6.5 by ascorbic acid, EGCG remained at 94.78% for up to 2 h, and remained at 86.04% after 4 h. There was no significant difference between pH 6.5 and pH 5.5. *In vitro* permeability of HBSS at pH 6.5 with ascorbic acid is expected to yield at least 85% of EGCG after 4 h of sampling (Fig. 1c).

In many studies, the stability of EGCG has been tested using cysteine, GSH, BSA, has, and ascorbic acid [14–17]. In one study, the stability of EGCG in the presence of three thiol-containing species [cysteine (Cys), glutathione (GSH) and 3-mercaptopentan-1-ol (3SH)] was followed under both neutral and acidic conditions. Both Cys and GSH increased the rate of EGCG oxidation at pH 4. At pH 7, only Cys was found to increase the rate of EGCG oxidation. In this experiment, cysteine was not suitable for the purpose of increasing the stability of EGCG under the conditions that can be tested in the cell [15]. Serum albumin is known to maintain stability through complex with EGCG. Using this property, coated BSA epigallocatechin gallate nanoparticles were prepared and high stability and permeability were confirmed in human intestinal epithelial Caco-2 cells [14]. In another study, it was reported that EGCG can form large water-soluble complexes with BSA or HSA and the complexes are stable to denaturation by detergents [18]. In this study, both BSA and HSA increased the stability of EGCG, and BSA showed higher stability than HSA. However, although viability could be improved in cell experiments, the stability of EGCG was not sufficient. Another study examined the effect of ascorbic acid and citric acid on the stability of green tea catechins (GTCs) as a mixture of EC, ECG, EGC, and EGCG, incubated in sodium phosphate buffer (pH 7.42). Ascorbic acid added to the incubation mixture significantly increased the stability of GTCs, whereas citric acid exhibited no effect. Four epicatechin derivatives examined demonstrated varying stability,

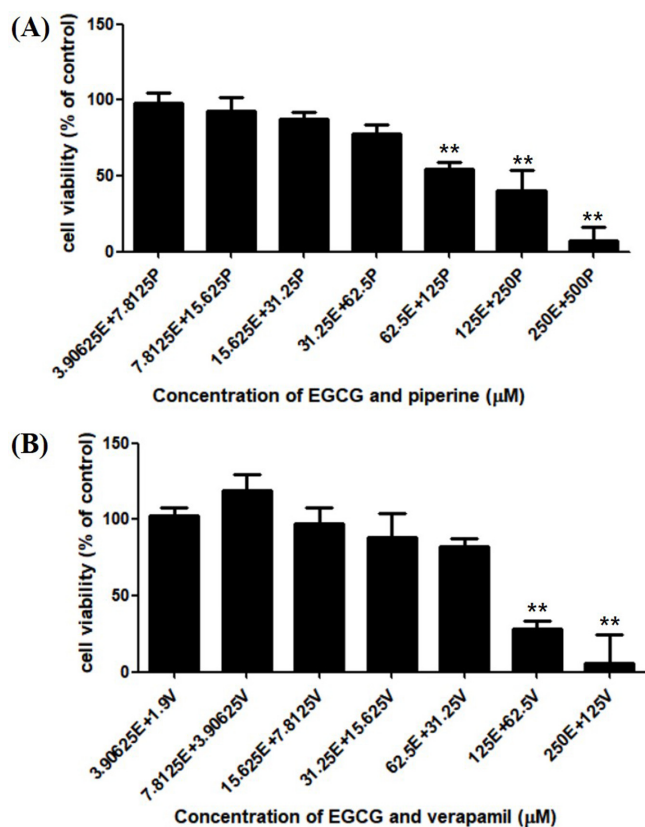
with EGCG and EGC being equally instable and EC and ECG being relatively stable. The addition of ascorbic acid significantly increased the stability of all four derivatives, particularly EGC and EGCG [17].

In our experiment, the pH-controlled experiments showed that the stability of EGCG was increased only when the pH was changed to 4.5–3.5. In the study of Song et al. [3], the stability of EGCG in niosomes of EGCG was also tested in HBSS buffer pH 7.4 using ascorbic acid (10 mM). The final pH is expected to be acidic; so, in our experiment, the smallest amount of ascorbic acid was tested to find the section that could be tested. As a result, when HBSS was treated to pH 6.5 using ascorbic acid, it was selected as a method to stabilize EGCG while enabling cell experiments. According to one study with a decrease in pH from 7.4 to 5.0, there was a 2.2-fold increase in the cellular accumulation of ECG and GTC [19].

In cell uptake experiments, changes in pH can affect the transport of material, so experiments at least in the range are important. In experiments that determine drug absorption and drug permeability through a Caco-2 monolayer, the pH of HBSS is recommended to be 7.4 but, depending on the type of experiment, the acid microclimate of the small intestine can be mimicked and the pH of the apical compartment can be adjusted to pH 6.5 [12].

### Cytotoxicity

In the permeability experiment, EGCG and piperine were mixed to be co-administered at high concentration (1:2), and the cytotoxicity in Caco-2 cells was measured. To measure their cytotoxic effects, EGCG and piperine were mixed up to 250 and 500  $\mu\text{M}$ , respectively, diluted in multiples of two, and administered at a minimum of 3.90625 and 7.8125  $\mu\text{M}$ . The cell viability of the highest concentration of EGCG and piperine was 7% ( $p<0.001$ ).



**Fig. 2** Viability of Caco-2 cells exposed to (A) EGCG and piperine (1:2), (B) EGCG and verapamil (1:0.5) for 4 h. Data points represent mean  $\pm$  SD ( $n=3$ ). \*Significantly different from control group,  $p < 0.05$ , \*\*significantly different from control group,  $p < 0.01$

The viability of the EGCG with piperine (7.8125 and 15.625  $\mu\text{M}$ , respectively) group was 92.95% with no significant change compared to the control. At EGCG and piperine concentrations of 31.25 and 62.5  $\mu\text{M}$ , cell viability was decreased to 77.41% (Fig. 2A). When verapamil was used as a positive control, the cell viability of EGCG 31.25  $\mu\text{M}$  and verapamil 15.625  $\mu\text{M}$  was 88.57%, with no significant change (Fig. 2B).

EGCG is a strong antioxidant and is characterized by its color reaction with XTT reagent. While going through the process of washing several times, it is important that cells are conserved, as they can easily fall from the plate. The treatment capacity was determined at a viability level of about 80% and the survival rate compared to the control was measured by cell counting for each dose. The survival rate for the EGCG and piperine doses used in the permeability test was  $97 \pm 3\%$ . The survival rate in the group receiving EGCG and verapamil was  $81 \pm 8\%$  (Table 1). The dose used in the XTT cytotoxicity experiment showed no cytotoxic effect on Caco-2 cells.

According to previous research, EC, EGCG, and GA (gallic acid) were found to be non-toxic at concentrations ranging between 100 and 250  $\mu\text{M}$  with a cellular survival rate of 70–80%, whereas a 60% cytotoxicity occurred at a concentration of

**Table 1** Survival percent of Caco-2 cells after 24-h treatment with EGCG and piperine

Treatment	Survival rate (%)
Control	-
EGCG (31.25 $\mu\text{M}$ )	$96 \pm 23$
EGCG and piperine (31.25 and 62.5 $\mu\text{M}$ )	$97 \pm 3$
EGCG and verapamil (31.25 and 15.625 $\mu\text{M}$ )	$81 \pm 8$

500  $\mu\text{M}$  for all flavonoids tested in Caco-2 cells [20]. In this experiment, we tested the toxicity of EGCG on Caco-2 cells with a sample of piperine (500  $\mu\text{M}$ ) and verapamil (125  $\mu\text{M}$ ) by applying the highest ratio of piperine and verapamil to be administered together with 250  $\mu\text{M}$  of EGCG based on these concentrations. When cell viability is lowered due to toxicity, the tight junction of the monolayer can be released. After the experiment, the tight junction was reconfirmed by checking the level of permeability with lucifer yellow.

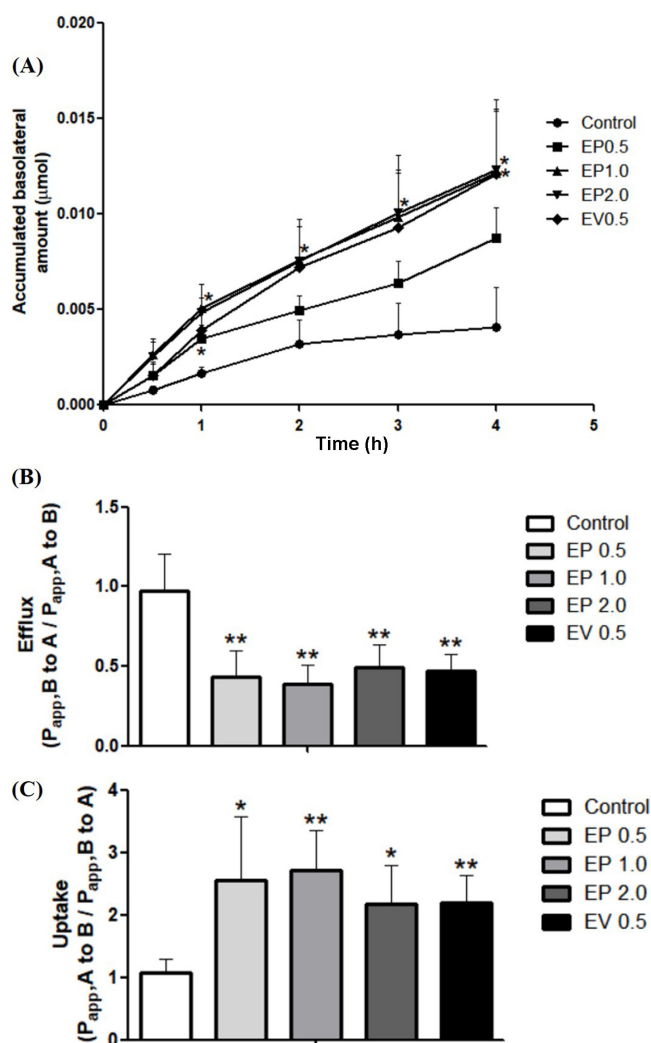
**EGCG accumulation on Caco-2 cell monolayer with piperine**

The EGCG uptake content, according to the piperine content, was tested in the Caco-2 cell monolayer. The level of EGCG uptake increased with time, and an increase in uptake showed in the piperine-treated group (0.5, 1.0, and 2.0 times of EGCG) compared to the control group. In particular, the uptake in the experimental group treated with piperine (2.0 times of EGCG) was confirmed to increase significantly after 1 h ( $p < 0.05$ ). This result was similar to the verapamil treatment group (Fig. 3a).

The transport of phenolic compounds to the cell is affected by a certain type of protein transporter, and hydrophilic compounds can be transported to the enterocytes by SGLT1. Glucuronides and sulfates of polyphenolic compounds in the small intestine are too hydrophilic to enter the cell by simple diffusion [21]. In this process, ATP-binding cassettes act, including multidrug resistance proteins (MRPs), breast cancer resistance proteins (BCRP, ABCG2), and P-gp. MRP2 is responsible for transporting polyphenols, such as EGCG, with glucuronide and sulfate to the membranes of enterocytes [22]. P-gp is also responsible for efflux transport.

**Transepithelial transport of EGCG in Caco-2 cells with piperine**

Little is known about absorptive and efflux transporters in relation to transepithelial transport of EGCG. However, glucuronides or sulfates make the material very hydrophilic for simple diffusion. Absorbed material are known to be effluxed by MRPs and P-glycoproteins. In this study, we also performed transepithelial transport assays to determine the effect of piperine on this mechanism. Simultaneous administration of piperine increased the Papp for basal to apical and apical to basal transport of EGCG. In the experimental group treated with piperine (0.5, 1.0, and 2.0 times of EGCG), Papp A to B tended to increase to  $3.741 \pm 2.14$ ,  $5.784 \pm 3.08$ , and  $5.864 \pm 3.05 \times 10^6$  cm/sec, respectively. In addition, Papp B to A showed a tendency to increase to  $1.731 \pm 1.57$ ,



**Fig. 3** Permeability of EGCG with piperine on Caco-2 cell monolayers. (A) Accumulated amount of EGCG in the basolateral chamber during the 4-h experiment. (B) Uptake is calculated as the ratio of apical-to-basal apparent permeability coefficient (Papp) to basal-to-apical Papp for 4 h. (C) Efflux is calculated as the ratio of basal-to-apical Papp to apical-to-basal Papp for 4 h. Data points represent mean  $\pm$  SD ( $n=3$ ). \*Significantly different from control group,  $p<0.05$ , \*\*significantly different from control group,  $p<0.01$ . EP0.5, EGCG 31.25  $\mu$ M and piperine 15.625  $\mu$ M; EP1.0, EGCG 31.25  $\mu$ M and piperine 31.25  $\mu$ M; EP2.0, EGCG 31.25  $\mu$ M and piperine 62.5  $\mu$ M; EV0.5, EGCG 31.25  $\mu$ M and verapamil 15.625  $\mu$ M

2.114 $\pm$ 0.988, and 2.772 $\pm$ 1.32  $\times 10^6$  cm/sec, respectively (Table 2). However, the increase to A to B was larger than the increase to B to A. Due to this effect, the net efflux decreased compared to the control. As a result, uptake (Papp A to B/Papp B to A) was significantly increased in the piperine co-administered group compared to the EGCG-alone group, and significant decreases were found in the efflux (Papp B to A/Papp A to B) in the 0.5-, 1.0-, and 2.0-fold treatment groups compared to EGCG ( $p<0.01$ ). The same phenomenon was observed in the glucuronidase

**Table 2** The apparent permeability coefficients (Papp) and efflux (Papp B to A/Papp A to B) of EGCG treated with piperine

Treatment	Papp ( $\times 10^{-6}$ cm/s)		Efflux
	A to B	B to A	
Control	1.907 $\pm$ 1.99	1.700 $\pm$ 1.85	0.970
Piperine 0.5 (15.625 $\mu$ M)	3.741 $\pm$ 2.14	1.731 $\pm$ 1.57	0.435
Piperine 1.0 (31.25 $\mu$ M)	5.784 $\pm$ 3.08	2.114 $\pm$ 0.88	0.389
Piperine 2.0 (62.5 $\mu$ M)	5.864 $\pm$ 3.05	2.772 $\pm$ 1.32	0.494
Verapamil 0.5 (15.625 $\mu$ M)	5.349 $\pm$ 4.19	2.234 $\pm$ 1.55	0.472

inhibitor and the non-selective first generation P-gp inhibitor verapamil group (Figs. 3B, C).

In this experiment, we evaluated the permeability of EGCG on a Caco-2 monolayer by treating it with the bioenhancer piperine, which is known to have glucuronidase and P-gp inhibitory effects. As a result, it was confirmed that the Papp from apical to basal increased the piperine level in concentration-dependent manner. This showed a clear concentration dependence compared to the Papp from basal to apical, suggesting that the glucuronidation of EGCG was inhibited and the absorption increased without a change to the hydrophilic form. These findings correlate with those of other studies where piperine improved the bioavailability of (-)-epigallocatechin-3-gallate in mice. Glucuronidation of EGCG in the hepatic and small intestinal microsomes of mice was measured by piperine concentration. In small intestinal microsomes, glucuronidation decreased significantly with piperine concentration, at 100 and 500  $\mu$ mol/L, piperine inhibited small intestinal glucuronidation of EGCG by 40% and 60%, respectively [8].

In addition, piperine is known as an inhibitor of P-gp, which is expected to have an efflux inhibitory effect on EGCG. It is reported that the efflux mechanism of EGCG contains not only P-gp but also MRP2 [23,24]. Further investigation is required to determine how efflux of EGCG can be inhibited. Verapamil was also observed in the effects of EGCG on the pharmacokinetics of verapamil in rats. When the 0, 2, and 10 mg/kg of EGCG were administered, the AUC of verapamil was increased to 280 $\pm$ 80.0, 488 $\pm$ 112, and 591 $\pm$ 137, respectively. The presence of EGCG, rather than the difference in dose, seems to promote the absorption of verapamil, which was observed by CYP3A and P-gp inhibition of EGCG [25].

Piperine increased the permeability of EGCG in our Caco-2 cell monolayer. This suggests that piperine has the effect of inhibiting EGCG glucuronidation and efflux. Ascorbic acid treatment was an effective method for the permeability assay of EGCG *in vitro*. It is expected that *in vitro* methods may be more conveniently used in developing functional foods or drugs using EGCG in the future; however, more in-depth mechanistic studies are needed to determine the exact uptake and efflux mechanisms. These findings of this study suggest that the addition of piperine to EGCG containing food or drug should improve the bioavailability of EGCG, demonstrating the potential for more effective functions in

vivo, enhancing the practical efficacy with low doses. The EGCG would actually remain in the forms of EGCG or further metabolites by metabolic enzymes in human body, where the bioavailable forms of the compounds may be elucidated by further studies in vivo and in human clinical trials.

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