Epicatechin-Copper (II) Complexes: Damage of Small Intestinal Epithelium

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Abstract: Four epicatechins [(-)-epicatechin (EC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCg)] and their corresponding copper complexes were compared with regard to their effect on the viability of Caco-2 colon cancer cells in vitro, measured by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. The viability of Caco-2 cells exposed to EC (1 mM), ECg (1 mM) or EGC (1mM) respectively, for 30 min, was comparable to that of the saline control group, while EGCg (1 mM) apparently enhanced cellular activity. In contrast, the cells treated with epicatechin-copper complexes were killed. Bivalent copper (1 mM), in similar conditions, did not affect the cells. No cell leakage or other histological differences were observed, implying a rapid cell death. The suggested mechanism of killing is by OH radical attack, produced in the presence of epicatechin-copper complexes, but not in the presence of either of the epicatechins or copper alone. The reaction sites are discussed.

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1 Introduction

Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics, found ubiquitously in plants. Over 4,000 different flavonoids have been
identified within the major flavonoid classes that include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones [1].

Flavonoids are part of the human diet. They are absorbed from the gastrointestinal tract and are excreted either unchanged or as flavonoid metabolites in the urine and feces. Flavonoids are potent antioxidants [2], free radical scavengers [3], and metal chelators [4], and inhibit DNA strand breakage [5] as well as lipid peroxidation [6].

Epicatechins (Fig. 1a), a subclass of flavonoids, are the major active components of green tea [7, 8, 9]. Upon ingestion, they reach the intestinal lumen where they come in contact with various metals like iron (Fe\(^{2+}\)), zinc (Zn\(^{2+}\)) or copper (Cu\(^{2+}\)).

Copper is an essential micronutrient and a required component of many enzymes including: lysyl oxidase, cytochrome c oxidase, and superoxide dismutase. It is contained in foods such as beef meat, poultry liver, chocolate, lobster etc. [10]. Yet, cellular copper concentrations above a certain level are harmful. For instance, Wilson’s disease is a toxicosis condition in which copper absorbed from the intestine builds up in the liver, due to the liver’s inability to release it into the bile. Thus, copper injures liver tissue and is eventually released into the bloodstream, which carries it throughout the body, leading to damage in the kidneys, eyes and brain. Currently, this disease is treated with lifelong use of drugs that facilitate the removal of copper (ex. D-penicillamine or trientine hydrochloride) and the administration of zinc that induces the expression of metallothionein, a carrier protein for heavy metal ions like copper [11].

The understanding of copper homeostasis has become increasingly important in clinical medicine as copper was suggested to be involved in the pathogenesis of some neurological disorders such as Alzheimer’s disease, motor neuron diseases, and prion diseases [12, 13]. Consequently, interest has been shown in finding nonmetal chemicals that may alter intestinal copper metabolism and thus help alleviate copper toxicity.

Catechins, apart from their capacity to enhance the induction of metallothionein and other antioxidant proteins in intestinal cells [14, 15, 16, 17, 18], also have direct antioxidant power through their ability to chelate copper [19] (Fig. 1b) and have, therefore, been sought as a means to remove copper from the intestinal tract [20, 21].

However, recent evidence has shown that catechins can also act as pro-oxidants, depending on the redox state of their biological environment [22]. The interactions between catechins and metal ions [21, 23, 24] may play a critical role in catechin-induced in vivo oxidative damage since they can generate reactive oxygen species, therefore causing oxidative stress that has a pivotal role in apoptosis.

Given the high bio-availability of catechins in the intestine [25], the purpose of this study is to investigate the action of the four major tea epicatechins in the presence of cupric ions (Cu\(^{2+}\)), on Caco-2 cells-a well accepted model for intestinal absorption [26].

The assay used in our study is based upon the dehydrogenase system of the intact target cells, which will normally convert an artificial substrate, MTT, into formazan (blue) that can be measured spectrophotometrically [27, 28]. In principle, if the epicatechin-copper complexes generate enough reactive oxygen species to initiate damage, the cells will have less or no ability to produce formazan blue [29].
2 Materials and methods

2.1 Reagents

Stock aqueous solutions of 5 mM epicatechins (epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg), Funakoshi, Japan) were made using sterile purified water (Mitsu-Ebitsu, Japan). Copper Sulfate was bought from Nakarai Chemicals Ltd. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo (Japan); dimethyl sulfoxide (DMSO), 99.9% (spectrochemical grade) was a product of Wako (Japan); poly-L-Lysine hydrobromide, M.W. 70-150 kDa, was bought from Sigma-Aldrich.

Dulbecco’s Modified Eagle Medium (DMEM, Cat. No. 11995), containing high glucose 4500 mg/mL, L-glutamine 584 mg/L, phenol red 15 mg/L, sodium pyruvate 110 mg/L, pyridoxine hydrochloride 4 mg/L, CaCl$_2$ (anhyd.)200 mg/L, NaH$_2$PO$_4$ 125 mg/L, NaHCO$_3$ 3.7 g/L, was purchased from Gibco (Japan), as well as the antibiotic mixture Penicillin-Streptomycin-Neomycin (PSN) and Trypsin-EDTA (0.05% Trypsin, 0.53 mM EDTA·4Na). Fetal Bovine Serum (FBS; Lot. No. 1108727) was a product of Gibco (USA). Physiological saline (100 mL bottles) was bought from Otsuka (Japan).

We used an inverted microscope (Eclipse TE300) from Nikon (Japan), as well as an UV-VIS-NIR Scanning Spectrophotometer (UV-3100PC) from Shimadzu, with 1-cm pathlength quartz spectrophotometer cells from GL Sciences Inc., all products of Japan. The UV-VIS Multiskan Multisoft microplate reader used was furnished by Labsystems (Finland).

2.2 Cell line

The American Type Culture Collection Caco-2 cell line from master stock (cryopreserved at generation 38) was expanded in Petri dishes, using standard culture medium DMEM, 10% FBS and 1% PSN antibiotics, at 37°C, 5% CO$_2$, 95% air, 100% relative humidity. Adherent cell monolayers approaching 90% confluence were harvested with trypsin-EDTA, counted and dispersed within poly-L-Lysine coated 24-well culture plates, at a low density of 7 x 10$^4$ cells/mL (1 mL/well). Media was changed in the first day after seeding, then every other day. All experiments were carried out on the seventh day after seeding. Culture plates were inspected under microscope and only those wells showing a smooth, well-attached monolayer were used. Passages 42 to 46 subsequently followed epicatechin treatment and tetrazolium assay.

2.3 Incubation of cells with catechins and copper sulfate

The experiments were designed at 1 mM aqueous solutions of epicatechins, close to a physiological concentration, as derived from the standard infusion model of tea [30, 31]. Therefore, after the cells were washed with 3 x 1 mL prewarmed saline (37°C), the neces-
sary volume of saline was added, followed by 5 mM stock epicatechins aqueous solutions, to a final concentration of 1 mM. The copper complexes were formed by subsequently adding copper sulfate 10 mM to a final concentration of 1 mM/well. The final volume of each well was 1 mL in all experiments. The controls were incubated saline alone.

The final experimental incubation time of 30 minutes was chosen after calibration experiments were run from 15 min up to 4 h. Following incubation, the reaction medium was removed from wells by slow aspiration through a blunt 18-gauge needle, the cells were washed with 3 x 1 mL prewarmed saline, then submitted to the MTT assay.

2.4 MTT Assay

The methodology described below represents a modification [28] of the original 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay described by Mosmann [27].

MTT stock solution (7.5 mg MTT/mL in sterile water) was stored at 4 °C for a maximum of 1 month. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:30 (v/v) in physiological saline. Under standard assay conditions, after incubation with/without epicatechins and/or copper, cells were washed of reaction medium with prewarmed saline solution (3 x 1 mL/well). 1 mL of MTT working solution was added to each well and cultures were incubated for 45 min at 37 °C. Following incubation all superantant was removed from wells by slow aspiration through a blunt 18-gauge needle and monolayers were washed with prewarmed saline (3 x 1 mL/well). Cell monolayers were microscopically inspected for adherence and culture wells containing detached cells were discarded. The remaining monolayers were tritutated with 1 mL DMSO/well. Following thorough formazan solubilization (trituration by pipette and vibration on a plate shaker), 200 µL solution from each well were transferred to a 96-well plate. UV absorbances were read at 492 nm, using a microplate reader.

In principle, the viable cell number/well is directly proportional to the production of formazan which, following solubilization, is measured spectro-photometrically.

2.5 UV Spectra

Absorbance spectra of formazan reagents as well as cell-generated formazans were measured with a UV/visible scanning spectrophotometer. Samples were placed in 1-cm path-length quartz cells. Freshly prepared material was analyzed in dual beam mode with 2-nm slit width, at 200 nm/min, 1Å threshold, and 0.15s response.

To avoid false-negative endpoints, separate standard configuration of blank wells (lacking cells) were prepared, which permit visual and spectrophotometric detection of chemical MTT reduction as well as a means to measure absorbance contributions from chromogenic solutions. Incubation was performed in saline because epicatechins in phosphate buffer give false positive readings for MTT assay[32]. It has been suggested before that
some oxidant compounds may interact with the MTT assay [27]. Experiments were performed in triplicate with at least 3 different samples/experiment and are expressed as mean ± SD. Statistical comparisons were made by ANOVA followed by an unpaired (homoscedastic) two-tailed Student’s $t$-test. Values of $p$ less than 0.01 were considered significant.

3 Results

Caco-2 cells incubated for 30 minutes in saline with different epicatechin-copper complexes, as well as epicatechins or copper alone, have been subsequently checked for viability using the MTT assay. The viability is expressed as the ability of forming purple formazan, which absorbs light at 492 nm. The full spectrum of the MTT/ DMSO solutions has been recorded at the end of the cellular assay (Fig. 2) and it showed that the optimal wavelength for the automated reading of the formazan absorbance is 492 nm. The allure of the spectra is similar to that previously described in the literature [28, 33]. The DMSO lysates of epicatechin-copper treated cells did not show formazan absorbance peaks.

As described, in principle, the production of formazan is proportional to the viable cell number/well. In a preliminary study, the cells’ incubation time in saline (30 min), as well as the MTT incubation time (45 min) have been selected after confirming the method’s linearity as shown in Fig. 3.

The results are summarized in Fig. 4. They clearly demonstrate that, compared to the control group, neither the four epicatechins (1 mM) nor copper (1 mM) cause a decrease in the viability of Caco-2 cells ($t$-test, $p_{\text{control–copper}} = 0.025$, $p_{\text{control–EC}} = 0.971$). In contrast, the presence of any of the four epicatechin-copper complexes (1 mM) considerably decreases the cells’ viability, as demonstrated by the significant drop in the production of formazan by all the complex-treated cells (ANOVA, $p_{\text{four complexes}} = 0.39$; $t$-test, $p_{\text{control–complex}} < 0.001$).

Although it has been reported that 1mM epicatechins induce cell death [34], in our study the viability of cells treated with 1 mM of EC, ECg and EGC, respectively, is similar to the control ($t$-test, $p_{\text{control–EC}} = 0.97$, $p_{\text{control–ECg}} = 0.024$, $p_{\text{control–EGC}} = 0.088$; or ANOVA, $p_{\text{control,EC,ECg,EGC}} = 0.041$). Moreover, the activity of those cells treated with EGCg is significantly larger than the control ($t$-test, $p_{\text{control–EGCg}} < 0.001$).

From a morphological point of view it is also clearly visible on the photos taken under the microscope that the cells treated with the epicatechin-copper complexes remain unstained (photos 4, 6, 8, 10 in Fig. 5), while the control and those treated only with epicatechins or copper alone appear stained (photos 1-3, 5, 7, 9 in Fig. 5). Therefore the latter are active. Moreover, from the general aspect of the cells under microscope, it can be inferred that the death is relatively sudden, and the loss of dehydrogenase activity precedes the membrane permeability, since no apparent morphological changes (cell swelling/shrinkage or cellular debris) are visible [35].
4 Discussion

4.1 Why MTT?

The tetrazolium salt in our assay functions as an electron acceptor from substrates or from some intermediate electron carrier. MTT is one of the most easily reduced tetrazolium salts [36]; on mild reduction (Fig. 6) MTT renders an intensely purple colored and water-insoluble formazan ($\lambda_{\text{max}}$ 490-560 nm [33], broad spectral absorbance band; $\varepsilon_{\text{max}}$ 51,000 $\text{M}^{-1} \text{cm}^{-1}$).

Moreover, being a mono tetrazolium salt, contamination of half-reduction products is not possible. Other physical and chemical characteristics of MTT such as small molecular size, good amphipathic capability, high stability to light exposure, and linear kinetics over a wide range of concentrations, make it most suitable for quantitative studies.

MTT’s intracellular conversion into formazans depends on the NADH and NADPH dehydrogenases [37]. Thus, it is an indicator of cellular metabolic activity.

However, MTT is not membrane permeable and it is taken up into cells through endocytosis. Reduced MTT-formazan accumulates in the endosome/lysosome compartment and is then transported to the cell surface through exocytosis [37]. The MTT assay as an estimate of cell viability actually measures endocytosis and exocytosis, a fundamental feature of most living cells (except the mature red blood cells). The number of intracellular MTT-formazan containing vesicles as well as the rate of exocytosis show tremendous differences among various cell types. Therefore, although the mechanism of MTT reduction applies to all mammalian cells, it should be taken into consideration that factors affecting either the endocytosis of MTT or the exocytosis of MTT-formazan will be able to affect cellular MTT assay results [38].

It has been reported that flavonoids can inhibit MTT-formazan exocytosis [38], therefore our results showing slightly more formazan in the EGCg treated group, compared to the control group, do not necessarily imply that EGCg has induced enzymatic changes at the NADH/NADPH level. The augmented values of formazan detected could be due to a possible specific mechanism of exocytosis inhibition.

5 Mechanisms of flavonoid action

Depending on the cells involved and the effects sought, different in vitro and in vivo studies have provided a variety of orders of activity for epicatechins [39]. A recent study on simulated digestion of green and black teas, in which epicatechins’ antioxidant capacity on the intestinal juices was determined, has found epigallocatechins to be more effective than epicatechins [31]. In our study, epigallocatechin gallate significantly enhanced the activity of Caco-2 cells, while epicatechin, epicatechin-gallate, and epigallocatechin did not show boosting effects. On the other hand all four epicatechin-copper complexes studied affected the cells, killing them.

There are doubtless many mechanisms by which epicatechins interfere with active
cells. In order to account for the antioxidant property of flavonoids in general, various explanations were proposed, ranging from capability to interrupt radical chain reactions due to direct abstraction of hydrogen from catechol groups to flavonoids’ intracellular ability to induce the expression of antioxidant proteins.

Interestingly, however, a structure-activity analysis indicated that the same features which are important to mutagenicity are also significant to antioxidant activity [40].

The metal-chelating property of catechins was, until recently, thought to be one of the mechanisms responsible for the anti-oxidant effects of these compounds in vivo. However, the autooxidation of catechins has been lately thoroughly investigated and has been shown to produce H$_2$O$_2$, the process being catalyzed by copper [24]. Other studies also showed that, in the presence of copper, epicatechins produce hydroxyl radicals, enhancing lipid peroxidation [22, 41]. Kimura et. al. revealed that the catechin-copper complex has oxidant capacity, since it kills bacteria [23]. The mechanism proposed (Fig. 7) involves molecular oxygen that interacts with the complex, near the cell membrane, to produce free radicals, which are toxic for the cells. It is likely that this is also the mechanism by which the intestinal cells in our study were killed.

However, a mechanism by which epicatechins deliver copper inside the cell, where the metal initiates oxidation, cannot be yet ruled out. Flavonoids are transported through the intestinal cell membrane as phenolic glucosides and galactosides by sugar transporters [42, 43]. However, sulfation- not glucuronidation, seems to be the major metabolic pathway for EC in humans [44]. Epicatechins, in particular, are transported by the MRP2 transporter, expressed in the apical membrane of Caco-2 cells [45, 46]. The four epicatechins used in our study possess the key structure for DNA cleavage in the presence of cupric ion, as determined by Hayakawa et. al. [47]. Their structure is also characterized by the presence of the major flavonoid copper-chelation sites, as suggested by Brown et al. [48].

Catechin/catechin self-association in a series of loosely bound low-energy complexes has been established by NMR NOE experiments and verified by MacroModel molecular complex conformational search methods [49, 50]. It is likely that epicatechins form clusters, thus enhancing copper fixation.

Therefore, a mechanism by which catechin-copper complexes transport copper through the cellular membrane, delivering it to the cytosolic enzymes and initiating damage from inside the cell, could be possible.

In summary, epicatechins, a subclass of catechins contained abundantly in tea and usually known as antioxidants, have shown oxidant properties against Caco-2 cells in the presence of copper (II), leading to rapid energetic cell death, as suggested by the morphological observations as well as the MTT assay used to determine the rate of viability. Since the presence of catechins inside the cell has already been demonstrated, a mechanism by which catechin-copper complexes transport this metal through the cellular membrane, delivering it to the cytosolic enzymes, cannot yet be ruled out.
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(-)-Epi-form (2R, 3R)
EC  (-)-Epicatechin: $R_1 = R_2 = H$;
EGC  (-)-Epigallocatechin: $R_1 = OH, R_2 = H$;
ECg  (-)-Epicatechin gallate: $R_1 = H, R_2 = X$;
EGCg  (-)-Epigallocatechin gallate: $R_1 = OH, R_2 = X$;

**Fig. 1** Chemical structures of: a—epicatechins; b—epicatechin-copper complexes.
Fig. 2 Full spectrum of various MTT/DMSO solutions pertaining to cells incubated for 30 min with copper sulfate, EC, ECg, EGC and EGCg, 1 mM respectively. Control group was incubated only in saline. MTT incubation time was 45 min.
Fig. 3 Calibration curve for MTT assay on Caco-2 cells. ■ Cells were washed of medium, then directly submitted to the MTT assay; ● Cells were washed of medium, incubated for 4 h in saline, then submitted to the MTT assay.
**Fig. 4** Activity (viability) of Caco-2 cells, expressed as absorbance of MTT formazan at 492nm. Cells were incubated for 30 min with 1 mM EC, ECg, EGC, EGCg, and four epicatechin-copper complexes and copper sulfate respectively, compared with the control group (saline only).
Fig. 5 Caco-2 cells stained with MTT, after incubation for 30 min with 1 mM of various epicatechins. 1, Control; 2, Cu (II); 3, EC; 4, EC+Cu (II); 5, ECg; 6, ECg+Cu (II); 7, EGC; 8, EGC+Cu (II); 9, EGCg; 10, EGCg+Cu (II); magnification x 200.
Fig. 6 Reduction of MTT in vivo.
Fig. 7 The mechanism of substrate oxidation by epicatechins and copper.