Uptake and distribution of catechins in fetal organs following in utero exposure in rats

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BACKGROUND: Although catechins are known to be powerful antioxidants, no reports have shown their transport to fetal organs. We investigated the distribution of catechins in fetal rat organs after maternal exposure to green tea extract (GTE). METHODS: GTE (550 mg/kg) or water was fed orally to pregnant dams at 15.5 days of gestation, the dams were sacrificed and fetal organs were dissected 0, 0.5, 1, 2, 3, 5, and 8 h later. Catechins and catechin gallates were determined by high-performance liquid chromatography (HPLC) after solid-phase extraction. RESULTS: In the GTE-treated group, catechins were detected in most of the fetal organs studied, including the brain, eyes, heart, lungs, kidneys and liver but not in the control group. The first peak times (T max) were about 0.5–1 h. The maximum concentrations (C max) of catechins in the fetal eye were about 2–10 times higher than in the other organs, ranging from 249 pmol/g for epicatechin (EC) to 831 pmol/g for epigallocatechin gallate (EGCG). Catechin gallates were generally more readily taken up by fetal organs than catechins. EGCG had the highest level of uptake according to area under the curve (AUC) plots and the highest C max in all organs. CONCLUSIONS: Various fetal organs had low but significant levels of catechins after GTE intake by the dams, and organ levels were found to be related to catechin structure. EGCG could be a potential candidate for antioxidant supplementation of the fetus in utero.

Key words: antioxidant/catechins/distribution/fetal tissues/green tea

Introduction
Tea is the commonest beverage in the world after water (Wang et al., 1998; Ahmad and Mukhtar, 1999; Yang and Landau, 2000). Because of its health benefits and wide safety margins (Mitscher et al., 1997), tea has been extensively studied for prophylaxis against cardiovascular diseases (Yang and Landau, 2000). Tea also exhibits anti-inflammatory, antimicrobial, anti-mutagenic and anticarcinogenic properties (Khan et al., 2006). These medical effects are mainly attributed to its antioxidative properties (Mitscher et al., 1997; Wang et al., 1998; Ahmad and Mukhtar, 1999; Yang et al., 1998; Yang and Landau, 2000; Geetha et al., 2004). Green tea in particular contains high levels of antioxidative polyphenols, consisting mostly of catechins and catechin gallates (Mitscher et al., 1997). The essential constituents are (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GC), (–)-epigallocatechin (EGC), (–)-catechin gallate (CG), (–)-epigallocatechin gallate (EGCG), (–)-epicatechin gallate (EG) and gallocatechin gallate (GCG).

Oxidative stress can cause serious damage to both pre-term and near-term neonates (Rosenfeld and Davis, 1998; Saugstad, 1998). Large amounts of free radicals are generated during hypoxia/reperfusion processes associated with labour sometimes leading to asphyxia in the newborn (Saugstad, 1998; Johnston et al., 2001, Tastekin et al., 2005). These free radicals or reactive oxygen species can damage essential biomolecules, induce cell death and impair organ development (Saugstad, 1998; Khalil et al., 2006). The effects are especially serious in pre-term babies who have poorly developed antioxidative defence systems (Rosenfeld and Davis, 1998; Bracci et al., 2006). Neonatal complications resulting from oxidative stress include retinopathy, bronchopulmonary dysplasia, necrotizing enterocolitis and hypoxic-ischaemic encephalopathy (Saugstad, 1998; Tin and Wariyar, 2002). Administration of antioxidants around the time of birth can prevent mortality in asphyxiated infants although results are inconsistent (Fulia et al., 2001; Benders et al., 2006).

Many antioxidants have been used for prophylaxis against oxidative stress, including superoxide dismutase, glutathione reductase, catalase, vitamin E, vitamin C, melatonin and allopurinol (Rosenfeld and Davis, 1998). However, they each have disadvantages: enzyme treatments require injection, vitamin E and C provide antioxidative effects without additional protective effects such as catalytic ion chelation or they produce inconsistent clinical benefits (Mak and Neuton, 2001; Mooney et al., 2005; Sagar, 2006). Poston et al. (2006) reported no beneficial effect for high-dose vitamins C and E in pre-eclampsia,
Materials and methods

Materials

C, EC, GC, EGC, CG, ECG, GCG, EGCG, β-D-glucuronidase (G-0251), sulfatase (S-9754), ascorbic acid, uric acid and reduced glutathione (GSH) were from Sigma Chemical Co. (St Louis, MO, USA). Sodium dithionite (GSH) was obtained from RDH (Wunstorfer Str, Seelze). Waters (GSH) were from Sigma Chemical Co. (St Louis, MO, USA). Sodium sulfatase (S-9754), ascorbic acid, uric acid and reduced glutathione (GSH) were from Sigma Chemical Co. (St Louis, MO, USA). Sodium dithionite was obtained from RDH (Wunstorfer Str, Seelze). Waters Spherisorb ODS-2 (150 mm × 4.6 mm, 3 μm), linked with a Waters 600S high-performance liquid chromatography (HPLC) system consisted of a 626 pump, 717 autosampler, AF in-line degasser, linked with an eight-channel ESA CoulArray detector and an ESA column heater. The operation system controlling the pump and autosampler was a Millennium Ver. 3.2. CoulArray for Windows Ver. 1.04 was used for data acquisition. The analytical column was a Waters Spherisorb ODS-2 (150 mm × 4.6 mm, 3 μm), linked with a Waters spherisorb guard column ODS-2 (7.5 × 4.6 mm, 3 μm).

Experimental animals

Seven groups of pregnant Sprague–Dawley rats, each weighing about 300 g, aged 9 weeks and at 7.5 days of gestation, were obtained from the animal house of the Chinese University of Hong Kong. Ethics approval for the study was obtained from the Animal Ethics Committee of the University. Dams were housed at 25°C with a 12-h light and dark cycle with free access to Chow and water for 7 days. The dams were then fasted overnight and weighed before experimentation. Fetuses of 15.5 gestational day were studied because these were still in the organ developmental stage, whereas the organs were of sufficient size for microdissection. GTE tablets were powdered and suspended in 0.5 ml of water. Forty-two dams were randomly assigned to seven groups: viz. 0, 0.5, 1, 2, 3, 5 and 8 h. They were fed with 0.5 ml of 0.55 g/kg GTE suspension with feeding tube. Another seven dams were fed with 0.5 ml of water without GTE as negative controls for each time point. At 0, 0.5, 1, 2, 3, 5 and 8 h after treatment, the dams were anaesthetized [0.5 ml of ketamine 10% and xylazine 2% (9:1) mixture i.m.] and sacrificed. The dams in the 0-h group were sacrificed immediately after feeding and anaesthetization. The fetuses from each dam were surgically removed. The fetal organs (brain, eye, lung, heart, liver and kidney) were dissected out from 12 to 14 fetuses from each dam using a dissecting microscope. Each organ (e.g. kidneys) from fetuses from the same dam was pooled into 1.5-ml centrifuge tubes after thorough washing in ice-cold saline and were snap-frozen in liquid nitrogen and stored at −80°C. Organs were pooled to increase the amount of catechins for better quantification. Maternal blood was collected immediately after sacrifice. Plasma was obtained after centrifugation at 1100g at 4°C for 10 min as described in Chu et al. (2006).

Tissue preparation

Tissue preparation and analysis followed our previously validated method (Chu et al., 2004). Good linearity over 99.2% was obtained in the range 20–4000 ng/g. The coefficients of variance were <5%. Absolute recovery ranged from 62 to 96% with accuracy 92.5–104.9% and detection limit 5 ng/g. The fetal organs were weighed and homogenized in 0.25 ml of methanol/ethyl acetate (2:1) and 0.25 ml of 0.3 M sodium dithionite/0.1% (w/v) Na2EDTA on ice. After centrifugation at 10 000 g at 4°C for 10 minutes, the supernatant was purged by nitrogen to remove the organic solvents and reduce the volume to about 0.2 ml. Then, 0.25 ml of 0.4 M phosphate buffer (pH 6.8) and 20 μl of a mixture of β-D-glucuronidase (2500 U) and sulfatase (1 U) were added for digestion of conjugated catechins by incubation at 37°C for 45 min. The reaction was stopped by placing the test tubes in ice.

Extraction of catechins

Both conjugated and unconjugated catechins were extracted for total catechin measurement. Tissue homogenate samples were diluted with 1 ml 0.05 M phosphate buffer (pH 7.0) and applied to a 30-μg Waters HLB column conditioned by 1 ml of methanol and 2 ml of water. After washing with 2 ml of water, 1 ml of 0.05 M phosphate buffer (pH 3.0), 1.0 ml of 0.05 M phosphate buffer (pH 7.0) and 1 ml of 5% methanol, the column was vacuum dried. Ethyl acetate, 10 ml, was added to remove lipophilic matrix. After vacuum drying, 10 ml of methanol/ethyl acetate (2:1) at 35°C was used to elute catechins into a tube containing 20 μl of 2% ascorbic acid–EDTA to preserve catechins during elution. The eluate was vacuum evaporated and blown dry by nitrogen. The residues were dissolved in a mixture containing 10% acetonitrile and 0.06% (v/v) trifluoroacetic acid (TFA) in 0.05 M phosphate pH 3.0. After filtering through a 0.2-μm pore size polytetrafluoroethylene membrane filter, a 20 μl of aliquot was injected into HPLC.

Chromatographic conditions

Binary gradient elution was used. Mobile phase A contained acetonitrile, water and TFA 80:920:0.6 (v/v). Mobile phase B contained 0.1% (v/v) TFA in 80% acetonitrile.
methanol, acetonitrile, water and TFA 30:70:0:6 (v/v). Both solutions were adjusted to pH 2.5. The gradient program and chromatographic conditions were as previously described (Chu et al., 2004).

**Data analysis**

The catechin concentrations in fetal organs were analysed using the WINNONLIN software package (Professional version 4.01). Statistic analysis was evaluated using the Statistical Package for the Social Sciences 10.1. The mean was compared by non-parametric analysis using Kruskal–Wallis H method. Maximum peak time (T_{max}), maximum concentration (C_{max}) and area under the curve (AUC) were computed. Exposure of individual catechins was presented as normalized AUC calculated by the trapezoidal formula in terms of molarity (pmol × h/g). Relative AUC was determined to compare the exposure levels of catechins in each fetal organ in relation to the corresponding exposure levels of maternal plasma (Chu et al., 2006). The equation of relative AUC is as follows:

$$\text{relative AUC}_{0-8h} = \frac{AUC \text{ (fetal organ)}_{0-8h}}{AUC \text{ (maternal plasma)}_{0-8h}}$$

The unit of relative AUC is ml/g.

Catechin data from each fetal organ were then evaluated by non-compartmental models. To identify the average time a molecule stayed in a fetal organ, predicted mean residence time from zero time point to infinity (MRT_{inf}) was used. This figure was obtained from the predicted area under the first moment curve (AUMC_{inf} in pmol × h^2/l) divided by the predicted AUC from zero time point to infinity (AUC_{inf} in pmol × h/l).

**Results and discussion**

**Catechin exposure**

Figure 1 shows typical chromatograms of catechins detected in the dam’s plasma and in various fetal organs: catechins were well separated without obvious interference, and although many catechins existed in trace amounts, they were still detectable.

We used total catechins instead of free or conjugated catechins for analysis. There are many different forms of conjugation, and each conjugate has its own physicochemical and antioxidative properties. The major conjugates of catechins in plasma are 5-O-β-glucuronides that retain the antioxidative properties of their parent compounds (Harada et al., 1999; Kida et al., 2000). Measurement of both conjugated and unconjugated catechins (total catechins) should give a reasonable overview of distribution.

We employed MRT_{inf} instead of MRT_{0-8h} because some catechin concentrations remained high even at the 8th hour (Figure 2). Also, MRT_{0-8h} tended to underestimate the sustained trend and shorten the estimated retained period. Terminal slope (λz) after the peak was low (Table I), suggesting prolonged presence of the catechins in the tissues, and the apparent clearance (CI/F) was very large implying very low bioavailability (F). In our study, we used relative AUC, which is a better parameter for representing the overall exposure of a tissue during the study period because it takes the input plasma catechin levels into account.

In the control group, catechins were not detectable in any fetal organs (data not shown). In the study groups, great variation was found in the concentrations of catechins in different fetal organs (Figure 3). Some pharmacokinetic profiles exhibited several peaks during the 8-h study period. T_{max} for most catechins in most tissues was between 0.5 and 1.2 h with the exceptions of GCG and ECG in fetal liver (2.3 h) and GC in fetal lung (3.0 h) (Table I). A second peak was found at the 2nd hour in brain and liver and at the 5th hour in kidney and heart. The concentrations rebounded during the experiments in fetal brain, eye and liver. This phenomenon is commonly found in fetal pharmacokinetic studies on flavonoids (Chu et al., 2006; Soucy et al., 2006). The reason is unclear, but may be because of the active uptake by the fetus during the diurnal cycle or of the delay in placental transfer of polar metabolites such as glucuronides (Unadkat et al., 2004), which are then metabolized back to their parent compounds by the fetal liver after the first peak (Soucy et al., 2006).

Not all catechins were found in all the organs (Table I). C and GCG were not detected in fetal eye, and GCG was not detected in fetal lung. EGCG, GC, EGC and EC were always present in all fetal organs. The level of CG was too low for accurate determination and therefore is not reported here.
Figure 2. Pharmacokinetic profiles of catechin (upper panels) and catechin gallates (lower panels) in fetal organs after a single dose of 550 mg/kg of Sunphenon DCF-1 green tea extract administrated orally to pregnant rats. Data points represent the average (+SD) concentrations of each catechin at different time points from fetuses of six dams.
EGCG had the highest $C_{\text{max}}$ and normalized AUC in most of the organs except for fetal lung, indicating relative poor absorption in fetal lung tissue. The catechin gallate group had higher relative AUCs than the catechin group (Figure 3), indicating that the gallates were absorbed more readily than the catechin bases. This implies that catechin absorption may be controlled by selective placental transfer similar to many other hydrophilic xenobiotics (Unadkat et al., 2004). Comparatively high relative AUC for EGCG, ECG and GCG suggest that active transport mechanisms may be involved.

**Fetal organ distribution**

The fetal brain, eye and lung are particularly susceptible to hypoxia and oxidative stress. Therefore, it is essential to have adequate antioxidant activity in these tissues during pregnancy. It is also essential to know whether catechins can reach the heart, liver and kidney as these organs are important for distribution, metabolism and elimination. In this study, we found significant levels of EGCG in the fetal brain and eye and substantial levels of GC and EGCG in fetal lung (Table I). As EGCG is one of the most powerful antioxidants amongst the catechins (Rice-Evans, 1995), it was encouraging to find EGCG as the dominant catechin in fetal organs following oral intake of GTE by the pregnant dam.

**Fetal brain**

Although the blood–brain barrier prevents penetration of many drugs, moderate levels (10–80 pmol/g) of catechins were found in the fetal brain (Figure 2, Table I). EGCG was the most abundant catechin in the brain tissue ($C_{\text{max}}$ 76.1 pmol/g and AUC 244.5 pmol $\times$ h/g). Other catechins, GC, EGC, C, EC and ECG, remained at low levels in the fetal brain throughout the 8-h study period. $t_{1/2}$, MRT$_{\text{inf}}$ and Cl/F of GC, EGC and EC could not be determined because no declining trends were found. Cl/F was higher (117–4909 kg/h) in fetal brain than in other organs.

**Fetal eye**

Catechin concentrations rose rapidly but also dropped rapidly (Figure 2). The $C_{\text{max}}$ (249–831 pmol/g) and normalized AUC (525–2195 pmol $\times$ h/g) of most catechins were higher in the eye than in other fetal organs (Table I). EGCG and GC concentrations were maintained in the fetal eye at relative high level during the study period. The relative AUC of EGCG was significantly higher ($P < 0.05$) than that of other catechins in the eye (except GC) and was significantly higher than in other organs, suggesting an active absorption mechanism may be involved (Figure 3). EC was found to have a significantly lower $t_{1/2}$ and longer MRT$_{\text{inf}}$ ($P < 0.05$) in the eye than in other tissues suggesting that EC stayed in the eye for very long time. Catechin levels in the eye were 10 times higher than in the brain, suggesting better perfusion of catechins to the eye. It is not known why C and GCG did not penetrate into the eye as effectively as the other catechins. There may be carrier mechanisms that selectively gate which compounds pass into the eye (Naggar et al., 2005; Quintana-Hau et al., 2005) or some enzymatic reactions that selectively breakdown these compounds during their passage (Bodor et al., 1995).
Pharmacokinetic parameters of catechins in fetal organs after a single dose of 550 mg/kg of Sunphenon DCF-1 green tea extract administrated orally to pregnant rats

| Parameter | Value
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>GC</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>EGC</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>C</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>EC</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>EGCG</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td>GCG</td>
<td>2.4 ± 2.3</td>
</tr>
<tr>
<td>ECG</td>
<td>1.2 ± 0.8</td>
</tr>
</tbody>
</table>

Data show mean ± SD values. Non-parametric analysis using Kruskal–Wallis H method.

**P < 0.05, significant parameters by comparing different catechins in each fetal organ.**

**P < 0.05, significant parameters by comparing different fetal organs for each catechin.**

This difference in concentration was also found in a preliminary study of eye and brain tissues from the dams: the GC was maintained throughout the study period, while EGCG, epicatechin gallate; ECG, epicatechin gallate; GC, (-)-epigallocatechin gallate; GCG, (-)-gallocatechin gallate; heart, terminal slope; M_Plasma, maternal rat plasma; MRT_{inf}, mean residence time from time zero to infinity; T_{max}, peak maximum concentration time of prominent peak.

AUC, area under the curve integrate normalized to molarity; C, (+)-catechin; C_{max}, maximum concentration of the prominent peak; CI/F, apparent clearance; EC, (-)-epigallocatechin; ECG, epicatechin gallate; GC, (-)-epigallocatechin gallate; GCG, (-)-gallocatechin gallate; liver, terminal slope; M_Plasma, maternal rat plasma; MRT_{inf}, mean residence time from time zero to infinity; T_{max}, peak maximum concentration time of prominent peak.

Data show mean ± SD values. Non-parametric analysis using Kruskal–Wallis H method.

**P < 0.05, significant parameters by comparing different catechins in each fetal organ.**

**P < 0.05, significant parameters by comparing different fetal organs for each catechin.**

Developmental stage and its potential for providing antioxidant protection require further evaluation.

Fetal lung

In the fetal lung, GC and EGCG were the dominant catechins. GC was maintained throughout the study period, while EGCG concentration slowly decreased (Figure 2). T_{max} of GC was about 3.0 h, indicating delayed absorption, but with higher C_{max} (129 pmol/g) and AUC (768 pmol × h/g) (Table I). As
with the other organs, the concentrations of EGC, C, EC and ECG in fetal lung were low but detectable, suggesting they could remain in the tissue. \( \chi \), MRT$_{inf}$ and Cl/F of EGC, C and ECG could not be determined.

**Fetal heart**

The level of GC fluctuated greatly and also had a second high C$_{max}$ at the 5th hour (Figure 2, Table I). A substantial concentration of EGGC was found in the fetal heart. Other catechins were maintained at low levels, but both GC and C had significantly raised MRT$_{inf}$ (\( P < 0.05 \)).

**Fetal liver**

The kinetic patterns of catechins in the fetal liver were very similar to those in fetal brain (Figure 2) with higher C$_{max}$ (10–215 pmol/g) and AUC (15–594 pmol × h/g). Whereas the levels of EGC, C, EC, GCG and ECG were maintained at low levels (Table I), the MRT$_{inf}$ of all catechins was relatively short (1.0–12.1 h) compared with other organs, indicating the potential metabolism of catechins during this developmental stage of the tissue.

**Fetal kidney**

The fetal kidney had the second highest C$_{max}$ and AUC of EC and ECG after the fetal eye (Figure 2, Table I). T$_{max}$ of the first dominant catechin peaks was around 1 h in the fetal kidney. Most catechins were found in comparatively high concentrations in the fetal kidney, suggesting the excretion may not be well developed at this stage of gestation: most catechins except ECG and EGC are eliminated predominantly via the kidney, rather than in bile, as in the adult (Yang et al., 1998). MRT$_{inf}$ of EGC was long and at a high level, implying that EGC accumulated in the kidney or that the kidney could not excrete it effectively.

Although the absolute concentrations of individual catechins detected in fetal organs were low, they were higher than those reported in adult rats (Meng et al., 2002). Takizawa et al. (2003) reported that only ∼3% (429 nmol/l) of an ingested dose of 50 mg/kg of EGC was detected in adult rat plasma and <10 ng/g (2.98 pmol/g of EGC) was found in the liver and kidney when tea extract was given for 3 days.

Cherubini et al. (1999) found that a concentration of at least 5 μM polyphenols in human plasma was necessary to achieve significant protection against lipid peroxidation in vitro, but that ex vivo plasma samples obtained 3 h after ingestion of the equivalent of six cups of black tea demonstrated no increased resistance to lipid peroxidation. In our previous study in rats (Chu et al., 2006), plasma concentrations of EC, which has the highest C$_{max}$ amongst all catechin compounds, were about 11.4 μM following administration of about 63 mg/kg of EC. This dose was equivalent to about 10 cups of green tea for humans after correcting by allometric scaling (Gabrielson et al., 2000). However, even with this dose, fetal eyes only had a C$_{max}$ for EGCG of 0.8 μmol/kg, and the C$_{max}$ in other organs was even lower. Nevertheless, we cannot directly compare concentrations in adults with those in the fetus where the effective concentration may be much lower, as is the case with other drugs (Stanwood and Levitt, 2004). Also we cannot directly compare the tissue concentration to the plasma concentration. Synergistic effects from all catechins should also be considered. Catechins are methylated by placental enzymes (Zhu et al., 2000), and the antioxidant power of methylated compounds is reduced compared to that of the corresponding non-methylated forms (Meng et al., 2002). We need to verify whether these concentrations provide a useful antioxidant effect at the tissue and the cellular levels in vivo and investigate whether an increase in the dosage to the mother can achieve higher concentrations in the fetus.

EGCG provided the highest relative AUC in all fetal organs, indicating its placental transfer rate is the highest amongst the catechins. It is well known that EGCG also possesses the highest reducing potential amongst catechins, making it a potential candidate for antioxidant supplementation of the fetus.

**Conclusions**

In summary, we found that catechins administered orally to a pregnant rat were able to penetrate into the organs including brain, eye, lung, liver, heart and kidney with the highest tissue exposure levels occurring in the eye. The relative AUC of catechin gallates group were higher than the catechins group in general, suggesting the absorption and deposition could be related to structure. EGCG gave the highest AUC and relative AUC amongst fetal organs. It’s level can be maintained for reasonably long periods of time, and it is also the most powerful antioxidant amongst all the catechins, making it the most suitable candidate for further investigation as a prophylactic antioxidant in pregnancy. Nevertheless, results from animal studies cannot be directly applied to humans. Further toxicology studies on human pregnancy should be conducted.

**Acknowledgements**

We thank Taiyo Kagaku Co. Ltd for their generous donation of GTE tablets, Sunphenon DCF-1. We are indebted to Professor Zuo Zhong from the School of Pharmacy, The Chinese University of Hong Kong, for valuable advice. This study was supported by Hong Kong Special Administration Region Research Grant Council (CUHK4077/01M).
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Submitted on April 19, 2006; resubmitted on July 12, 2006 and August 3, 2006; accepted on August 9, 2006

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