High Risk of Embryo-Fetal Toxicity: Placental Transfer of T-2 Toxin and Its Major Metabolite HT-2 Toxin in BeWo Cells

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Though T-2 toxin is the most harmful mycotoxin to the fetuses, it remains unclear whether T-2 toxin and its major metabolite, HT-2 toxin, could pass the placenta into the fetus and which kind of placental transport is involved in the passage. To illustrate their placenta transfer mechanism, the uptake and efflux of T-2 and HT-2 toxins across apical membranes of placenta with BeWo cells as a model were studied at different temperatures, pHs, and in the presence of transporter inhibitors with a developed liquid chromatography-tandem mass spectrometry to determine the amount of toxins in both fetal and maternal sites. Higher unidirectional transport of T-2 toxin was observed in the apical-to-basolateral direction than basolateral-to-apical one, whereas HT-2 toxin exhibited similar transport rate from the 2 directions. The main ATP-binding cassette transporters had no effect on the efflux of 2 toxins. Initial uptake of T-2 toxin was sodium dependent and saturable, and the apical uptake was temperature dependent and enhanced under acidic condition. The apical uptake of T-2 toxin was inhibited by metabolic inhibitors and the organic anion and organic cation transporter inhibitors. These results suggested that an active transport mechanism was responsible for the uptake of T-2 toxin, whereas passive diffusion was the principal mechanism for HT-2 toxin transport in the placenta. Taken together, these data characterized the placental transfer of T-2 and HT-2 toxins. The present study offered new ways of reducing the risks of T-2 and HT-2 toxins to both mother and fetuses.

Key Words: embryo-fetal toxicity; mycotoxin; T-2 toxin; HT-2 toxin; BeWo cell model; placenta.

As a world-wide environmental contaminant, T-2 toxin is highly toxic to animals and human beings. Acute T-2 poisoning caused emesis, anorexia, chills, abdominal distension, abdominal pain, thoracic stuffiness, diarrhea, and growth retardation (Ueno, 1983; Wang et al., 1993). It was reported that T-2 toxin might cause human Kashin-Beck disease, which might result from grains contaminated with T-2 toxin and selenium deficiency (Sun et al., 2012). Furthermore, unlike fumonisin B1 (FB1) (Voss et al., 2001), T-2 toxin showed embryotoxicity and teratogenicity, causing significant maternal mortality, fetal death, fetal body weight loss, and grossly malformed fetuses in mice or rats (Hood et al., 1978; Sehata et al., 2005; Stanford et al., 1975).

It was reported that T-2 toxin–induced apoptosis existed in the developing mouse fetuses, suggesting that T-2 toxin might readily pass the placenta and directly affect the fetuses (Ishigami et al., 2001). Furthermore, it was found that T-2 toxin could be transferred to the fetus through the placenta after intranginal injection or PO administration of tritiated T-2 toxin in late pregnancy rats (Lafarge-Frayssinet et al., 1990). Because T-2 toxin is rapidly metabolized to HT-2 toxin in most species, it is necessary to investigate whether it is T-2 toxin or HT-2 toxin that induces direct toxic effects on fetuses. However, it is impossible to verify which one of these toxins that are transported across the placenta and accumulate in the fetus because the radiolabeling methods cannot distinguish prototypes and their metabolites.

The radiolabeling methods could be applied to the placental transfer (Heaton et al., 2008; Rytting and Audus, 2008; Utaguchi et al., 1999). However, extensive safety monitoring is needed when using the radiolabeling methods. The radiolabeled drug is difficult to synthesize, and it is usually much more expensive than “cold” compounds. In transport study to assess the relevant risk of mycotoxin exposure, it is necessary to develop and validate an accurate and suitable analytical method.
for mycotoxin identification and quantification. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has excellent characteristics and might be applied to determine the concentrations of toxins in the placental transfer. However, to our knowledge, no LC-MS/MS method has previously been developed to investigate the placental transfer of mycotoxins. Therefore, we built a LC-MS/MS method to simultaneously determine the micro or trace levels of T-2 and HT-2 toxins. Furthermore, the sample preparation steps and organic extraction reagents were optimized to fit for the detection.

It is known that ATP-binding cassette (ABC) transporter protein, organic anion transporters (OATs), and organic cation transporters (OCTs) play important roles in importing and effluxing xenobiotics into and out of the fetal circulation (Ganapathy and Prasad, 2005). However, the transport and uptake characteristics of T-2 and HT-2 toxins in human placental cells are still unknown. The potential role of these transporters in the transport of T-2 and HT-2 toxins was assayed using their specific inhibitors. BeWo cells are derived from human choriocarcinoma and exhibited characteristics resembling those of the third-trimester trophoblasts (Friedman and Skehan, 1979; Pattillo et al., 1968). It has been demonstrated that BeWo cells could be used as an in vitro model of the rate-limiting barrier for maternal-fetal exchange (Young et al., 2002). The purposes of the present study were to reveal the transectental transfer of T-2 and HT-2 toxins in transwell systems with BeWo cells. To study the transport kinetics of T-2 toxin and HT-2 toxin across the placenta, the developed LC-MS/MS method to analyze the toxins in fetal and maternal transport media was utilized. The results would reveal whether T-2 toxin and HT-2 toxin could pass placenta into the fetus and what kind of placental transport was involved in the passage. This may contribute to identify novel methods to protect fetuses from toxin exposure and thereby decrease the occurrence of potential teratogenic effects.

**MATERIALS AND METHODS**

**Chemicals and reagents.** T-2 toxin and HT-2 toxin were purchased from Sigma (St Louis, Missouri). Verapamil was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Carboxylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and cyclosporin A (CSA) were purchased from Enzo Life Sciences (Farmingdale, New York). Choline chloride (choline Cl), 4-aminophipuric acid (PAH), and tetraethyammonium chloride (TEA) were purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan). 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid, monohydrate (MES), and 3-(4,5)-dimethylthiazol(-2-yl)-3,5-diphenyltetrazoliumbromide (MTT) came from Amresco (Solon, Ohio). Lactate dehydrogenase (LDH) kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, PR China). All other chemicals including 4,4′-diodostilbene-2,2′-disulfonic acid (DIDS), MK571, Ko143 were purchased from Sigma-Aldrich. Cell culture reagents were purchased from Invitrogen (Invitrogen, Carlsbad, California). Other chemicals were obtained from standard sources and were of the highest quality available. HPLC grade water, prepared using a Milli-Q plus system (Billerica, Massachusetts), was used for the preparation of HPLC eluents.

**Cell culture.** The BeWo cell line (CCL-98) was obtained from the American Tissue Culture Collection (ATCC No. 58311226). The cells were routinely grown in Dulbecco’s Modified Eagle’s Medium: F12 4.5 g/l glucose supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin (10 000 U/ml)/streptomycin (10 000 mg/ml) solution, 4 mM glutamine and 1% nonessential amino acids, 5% penicillin (100 U/ml) and streptomycin (100 mg/ml) in 75-cm² flasks in an atmosphere of 5% carbon dioxide in air at 37°C. When the cells reached 80%–90% confluence, they were plated on 6-well dishes or 12-well transwell inserts. For the uptake study, BeWo cells were seeded on 6-well plates (Corning Costar, Acton, Massachusetts) coated with rat tail collagen type I (Sigma) at a density of 1.0 × 10⁵ cells/well. The culture medium was changed every 2 days of incubation for 7 days. For the transport study, the cells were seeded at the density of 1.0 × 10⁵ cells/cm² on polyethylene terephthalate transparent inserts (0.4 nm pore size inserts, 0.9 cm² growth area, Corning Costar, Cat. No. 3460), in 12 multiwell plates, to allow a synchronization of cell differentiation process.

**Characterization of BeWo cells as an in vitro placenta model.** The suitable cell density was investigated when cells were seeded in 24-well plates at a density of 1.0 × 10⁴ or 2.0 × 10⁴ cells/cm². The MTT assay was performed to assess the effects of cell density on BeWo cell viability from the second to eighth day. The MTT assay was performed as described previously (Garcia et al., 2012). Briefly, 20 µl of MTT (0.5 mg/ml in PBS), 2-h incubation at 37°C, and the reduced formazan reaction products were measured at 570 nm. Optical density values were used for the results of MTT assay.

To assess paracellular permeability, the flux of 300 µg/ml fluorescein was measured as described (Liu et al., 1997). Monolayers were excluded if the transepithelial electrical resistance (TER) values fell by more than 15% during the transport experiments, or if the apparent permeability coefficients (P_app) of fluorescein in the receiver compartment exceeded 1.0 × 10⁻⁵ cm/s.

The cell microvilli and tight junctions between cells were observed by using transmission electron micrograph (TEM) and scanning electron micrograph (SEM), respectively, to identify whether BeWo cells have formed tight and polarized trophoblastic layers. BeWo cells were seeded at a density of 1.0 × 10⁵ cells/cm² for 7 days on transwell or 6-well plate. Then, cell samples were prepared and observed by using SEM and TEM.

**Cytotoxicity assays.** The MTT assay was performed to assess the effects of T-2 toxin and HT-2 toxin on BeWo cell proliferation as described (Wang et al., 2012). Cells, seeded in 96-well plates (Corning Costar), at a density of 1.0 × 10⁴ cells/cm², were incubated, 24 h after seeding, with 10, 20, 40, 80, 200, and 400 nM of T-2 toxin and 100, 200, 400, 800, 1000, and 2000 nM of HT-2 toxin for 8 and 24 h, respectively. A possible direct cytotoxicity was checked by assaying the extracellular LDH release (Kao et al., 2012; Lison et al., 2008). Maximal LDH release was obtained by exposing the cells to 1% (vol/vol) Triton X-100. The reduced formazan reaction product was measured at 500 nm and LDH activity was determined as mU/ml. LDH release rate (%) was calculated by the following equation:

\[
\text{LDH release rate (\%) = } \frac{\text{LDH activity in medium}}{\text{LDH activity in medium + LDH activity in lysate}} \times 100\%
\]

**Transepithelial transport studies.** The BeWo monolayers, grown on tranwell filters in 6-well plates, were preincubated for 30 min at 37°C in Hank’s balanced salt solution (HBSS), containing 10mM glucose and stirred gently. Apical (AP) and basolateral (BL) chamber volumes were maintained at 0.5 and 1.5 ml HBSS (pH 7.4), respectively. T-2 toxin (200 nM) or HT-2 (400 nM) toxin was added to either AP or BL compartment for the study of AP-BL and BL-AP transfer for 6 h (Supplementary Figure 1). The effects of inhibitors on transfer were studied after a 30 min pre-incubation in the presence of metabolic inhibitor (1 mM NaN₃ with 50 mM 2-deoxy-d-glucose [2-DG]), or ABC transport inhibitors (20 µM MK571, 10 µM VSP, and 20 µM Ko143), respectively. Mycotoxins contents in AP and BL compartments were measured after a 6-h exposure. The concentrations of the inhibitors were based on the literature (Tep et al., 2007; Videmann et al., 2007) and the pretest. At the end of both the...
preincubation and the experiments, monolayer integrity was checked by measuring TEER using a milliccil ERS (Millipore, Molsheim, France).

**Uptake studies.** The BeWo cells at 80%–90% confluence were washed twice with HBSS (136.7 mM NaCl, 0.385 mM NaHPO₄, 0.441 mM KH₂PO₄, 0.952 mM CaCl₂, 5.36 mM KCl, 0.812 mM MgSO₄, 25 mM D-glucose), and 10 mM HEPES for adjustment to pH 7.4 or 8.5 or 10 mM MES for adjustment to pH < 6.5, and then the test solution containing T-2 toxin or HT-2 toxin was added. The pH of the test solution was 7.4 except in the pH-dependent uptake experiments. At the end of the incubation, the test solution was aspirated away, and the cells were washed with ice-cold HBSS 3 times. The effect of the well-known inhibitors of OATs (DIDS, PAH, and probenecid) and the inhibitors of OCTs (TEA and cimetidine) on uptake was studied after a 30-min preincubation in the presence of inhibitors, and the uptake was measured in the coinubation of mycotoxin and inhibitors. For quantification of mycotoxin (T-2 toxin or HT-2 toxin) uptake, the cells were suspended in Tris-HCl (pH 7.4) containing 1% Triton X-100, and the suspension was incubated at 4°C overnight. The accumulated concentrations of mycotoxin were determined by HPLC-MS/MS after the cells were lysated. Cellular protein was quantified using a protein assay kit with bovine serum albumin as a standard. In one series of experiments, an equivalent amount of choline Cl replaced sodium chloride in the HBSS and was used as a sodium-depleted buffer.

**LC-MS/MS conditions.** Analysis of T-2 toxin and HT-2 toxin was carried out using an API 5000 Quantum Access triple quadrupole mass spectrometer coupled with a Surveyor LC pump and an autosampler. The system was controlled by BioAnalyist software (Applied Biosystems/MDS Sciex joint venture). The separation was achieved by a Thermo Hyperil Gold C18 (150 × 2.1 mm, 5 μm). Mobile phase A was 10% acetonitrile, whereas mobile phase B was 90% water. A gradient elution system was used: from 95% water, 5% acetonitrile at time 0, isotropic during 7 min, to 60% water, 40% acetonitrile at 10 min, isotropic during 2 min. The flow rate was 0.2 ml/min and the injection volume was 10 μl. Multiple reaction monitoring (MRM) was performed on each of the analyte protonated molecular ions using the parameters: source voltage was 5.5 kV, capillary temperature 550°C, sheath gas (nitrogen) 60, auxiliary gas 55, Q1 peak width 0.70 atomic mass unit (amu), Q3 peak width 0.50 amu, collision gas (argon) 6 mTorr, scan width 1–2 amu, and scan time 0.3–0.5 s. Collision energies were set at the maximum for each transition and ranged from 23 to 31 eV. Positive MRM mode was used to quantify concentration at m/z 489.1→387.3 and 489.1→245.1 for T-2 toxin, m/z 447.0→285.0 and 447.0→345.4 for HT-2 toxin.

**Sample preparations.** About 100 μl transport sample was measured into a 5-mL polypropylene centrifuge tube. Then, 200 μl acetic ether was added, and the mixture was vortexed for 5 min. Followed by centrifugation at 5000 × g for 5 min at 4°C, the supernatant was collected and evaporated to dryness under a stream of nitrogen in a water bath at 50°C. The residue was reconstituted in 200 μl solution (10% methanol-90% water) followed by vortexing for 5 min and transferred 20 μl solution to autosampler vials for LC-MS/MS analysis. We compared methanol, acetonitrile, chloroform, ethyl acetate, or a mixture of each other at different volume as the extraction reagents. Finally, acetic ether was applied as the most efficient extraction reagent.

**Validation.** Linearity of LC-MS/MS was evaluated by taking matrix-matched (HBSS) standard solutions that were analyzed in triplicate. A multi-component standard solution was prepared by spiking T-2 toxin or HT-2 toxin into matrix blank solution separately to make the concentrations of T-2 toxin at 0.5, 1, 2, 4, 8, 20, 40, and 80 nmol/l, and HT-2 toxin at 2, 4, 8, 16, 80, and 160 nmol/l. Limit of detection (LOD) for an analyte in matrix was calculated with a signal-to-noise ratio of 3. Limit of quantification (LOQ) for an analyte in the matrix was established with a ratio of signal-to-noise ratio of 10. Precision (repeatability), expressed as relative standard deviation (RSD%), and recoveries were determined interday by analyzing spiked samples. Samples that were spiked with 1, 2, and 4 times the LOQ, each of 5, were analyzed on the same day. Samples that have been spiked with concentrations described above, respectively, were analyzed by an independent operator on 3 days, and the overall mean recovery and interday RSD values were calculated. It was evaluated by analyzing a blank sample and a blank sample spiked at the LOQ level.

**Data analysis.** The apparent permeability values ($P_{app}$) were calculated in all experiments according to the equation:

$$P_{app} = \frac{dQ/dt}{AC}$$

where $dQ/dt$ was the slope of the cumulative amount transported during the time course of the period studied. $A$ was the area of the inserts, and $C_s$ was the starting concentration.

Permeability direction ratios (PDR) were calculated according to the following equation:

$$PDR = \frac{P_{app(AP-BL)}}{P_{app(BL-AP)}}$$

where AP-BL was the apical-to-basolateral transport and BL-AP was the basolateral-to-apical transport.

The kinetics of T-2 toxin uptake was described by fitting to the following Michaelis-Menten equation according to Graph Pad Prime 5.0:

$$V = \frac{V_m \times [S]}{K_m + [S]}$$

where $V$ was the uptake rate, $V_m$ was the maximum rate of mediated uptake, and $K_m$ was the substrate concentration that resulted in half-maximal uptake.

Each experimental point represents the mean ± SD of 3–6 measurements. Statistical analysis was performed using the SPSS 13.0 package. Values of $p < .05$ were considered significant.

**RESULTS**

The Identification of BeWo Cells as the Placenta Model

Based on the cell growth characteristics, a stable and reliable BeWo cell model was established. The BeWo cells in transwell at a density of 1.0×10⁴ cells/cm² for 6–7 days could form an intact cell monolayer, and the TEER measured was higher than required and the $P_{app}$ of fluorescein showed a density barrier (Figs. 1A and 1B). The micrographs of SEM showed microvilli (Fig. 1C) and TEM showed the tight junction (Fig. 1D).

**Validation of the LC-MS/MS Method**

The representative chromatograms and the corresponding product ion scan spectra of T-2 toxin and HT-2 toxin were shown in Figure 2. No endogenous or extraneous peaks were observed interfering with the assay. The method exhibited satisfactory linear responses to T-2 toxin ranging from 0.5 to 80 nmol/l ($r = 0.9996$) and to HT-2 toxin ranging from 2 to 160 nmol/l ($r = 0.9994$), respectively. The LOD and the LOQ were 0.5 and 1.0 nmol/ml for T-2 toxin from the upper and lower compartment, respectively, and 2.0 and 4.0 nmol/ml for HT-2 toxin from the upper and lower compartment, respectively. Average recoveries of T-2 toxin and HT-2 toxin were within the range of 86.0%–97.6% with a coefficient of variation range between 2.5% and 5.0% (Table 1).
Influence of T-2 Toxin and HT-2 Toxin on Cytotoxicity

MTT test showed that T-2 toxin induced cytotoxicity in a dose- and time-dependent manner with a significant effect appearing at 200 nM for 8h and 10 nM for 24h (Fig. 3A). HT-2 toxin significantly inhibited the cell viability at 400 nM for 8h and 100 nM for 24h. The maximal LDH release was obtained by exposing the cells to 1% (vol/vol) Triton X-100. Under the same conditions, LDH leakage, the indicator of cell
damage, was not significantly modified by T-2 toxin or HT-2 toxin (Fig. 3B).

Transepithelial Transport of T-2 Toxin and HT-2 Toxin in BeWo Cells

The trans-trophoblast passage of T-2 toxin across the BeWo monolayer was found to be asymmetric with the permeability coefficient greater in the AP-BL direction than that in the BL-AP direction, as shown in Figure 4A, indicating that the processes might be mediated by active transport. The mean absorption (passage from AP to BL) rate of T-2 toxin after 3 h was 28.65% ± 1.60% of the initial dose, whereas the mean excretion (passage from BL to AP) rate of T-2 toxin was 3.83% ± 0.34% of the initial concentration with significant differences. The corresponding $P_{\text{app}}$ of AP-BL and BL-AP of T-2 toxin was $7.09 \pm 1.4 \times 10^{-6}$ cm/s and $4.32 \pm 0.6 \times 10^{-6}$ cm/s, respectively. The $P_{\text{app}}$ of AP-BL of T-2 toxin was about 1.64 times higher than that of the BL-AP. The $P_{\text{app}}$ values of HT-2 toxin in the AP-BL and BL-AP directions were $7.67 \times 10^{-6}$ and $5.62 \times 10^{-6}$ cm/s, respectively, and there was no statistically significant difference between them (Fig. 4B). In these conditions, no other T-2 toxin metabolites were detectable at both the AP or BL poles except that the added T-2 toxin or HT-2 toxin was assessed in the chromatography profiles after treatments of the media. The transports of T-2 toxin and HT-2 toxin were also investigated in both directions at different toxin concentrations ranging from 200 to 1000 nM and 400 to 2000 nM, respectively. The duration of incubation was then set at 3 h. The T-2 toxin and HT-2 toxin transport in the 2 directions were strictly proportional to the concentration over the entire range. Concentration dependence of T-2 toxin and HT-2 toxin saturation was not achieved even at a higher concentration (Fig. 4C).

As shown in Figure 4D, none of verapamil, an inhibitor of the P-glycoprotein (P-gp), or MK571, the inhibitor of multidrug resistance–associated proteins (MRPs), or Ko143, the inhibitor of the breast cancer resistance protein (BCRP) did affect the transport of T-2 toxin or HT-2 toxin in both AP to BL or BL to AP directions. However, T-2 toxin transport in the AP-BL was significantly inhibited in the presence of NaN₃.

### TABLE 1
Accuracy and Precision of T-2 Toxin and HT-2 Toxin for the Analyses at 3 Spiking Levels (Mean ± SD, n = 25)

<table>
<thead>
<tr>
<th>Spiked Concentration (nmol/l)</th>
<th>Mean Recovery ± SD (%)</th>
<th>RSD (%)</th>
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</thead>
<tbody>
<tr>
<td>T-2 toxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>89.9 ± 4.1</td>
<td>4.6</td>
</tr>
<tr>
<td>2.0</td>
<td>92.4 ± 3.6</td>
<td>3.9</td>
</tr>
<tr>
<td>4.0</td>
<td>96.0 ± 4.8</td>
<td>5.0</td>
</tr>
<tr>
<td>HT-2 toxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>86.0 ± 2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>8.0</td>
<td>95.1 ± 4.1</td>
<td>4.3</td>
</tr>
<tr>
<td>16.0</td>
<td>97.6 ± 2.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

As shown in Figure 4D, none of verapamil, an inhibitor of the P-glycoprotein (P-gp), or MK571, the inhibitor of multidrug resistance–associated proteins (MRPs), or Ko143, the inhibitor of the breast cancer resistance protein (BCRP) did affect the transport of T-2 toxin or HT-2 toxin in both AP to BL or BL to AP directions. However, T-2 toxin transport in the AP-BL was significantly inhibited in the presence of NaN₃.

### FIG. 3
Cytotoxicity assays of T-2 toxin and HT-2 toxin on BeWo cells. A, Cell viability in BeWo cells exposed for 8 and 24 h to T-2 toxin and HT-2 toxin concentrations from 50 to 500 nM and 100 to 2000 nM, respectively. Results are expressed as % of control cell viability (n = 6). Significant differences versus control were indicated by *p < .05 and **p < .01, respectively. B, LDH release rate of BeWo cells exposed for 8 h to T-2 toxin and HT-2 toxin concentrations from 50 to 500 nM. One percent Triton X-100 was used as the positive control (n = 6). Results are expressed as % of LDH in the medium and cells (n = 6). **p < .01 as compared with the blank control condition. Abbreviation: LDH, lactate dehydrogenase.
and 2-DOG (inhibitors of ATP production). The TEER of the monolayer was unchanged by the addition of samples to the transwell insert.

### Characterization of T-2 Toxin and HT-2 Toxin Uptake by BeWo Cells

T-2 toxin and HT-2 toxin were rapidly accumulated in BeWo cells (Fig. 5A). The uptake was linear initially and reached equilibrium at about 3 min. Therefore, all subsequent uptake studies and kinetic analysis were performed from data collected through 3 min. Figure 5B showed the relationship between the initial rate of uptake of mycotoxin and its concentration in the incubation buffer. The results indicated that the uptake of T-2 toxin was saturable. The uptake processes were analyzed according to equation 3. The kinetic parameters calculated for T-2 toxin uptake were a $V_{\text{max}}$ of $7.9 \pm 1.4$ pmol/mg protein/min and a $K_{\text{m}}$ of $946.7 \pm 281.7$ nM. Whereas, HT-2 toxin was non-saturable even at the high concentration.

Figure 5C illustrated the effect of incubation buffer pH in the range from 5.0 to 8.6 on T-2 toxin and HT-2 toxin uptake by BeWo cells. The rate of T-2 toxin and HT-2 toxin uptake decreased with increasing pH from an acidic to a neutral pH. These findings, together with the concentration dependence and sensitivity to metabolic inhibitors, strongly suggested that
FIG. 5. Characterization of T-2 toxin and HT-2 toxin uptake by BeWo cells. A, Time-course characteristics of T-2 toxin and HT-2 toxin uptake by BeWo cells (n = 6). B, Concentration-dependent characters of T-2 toxin and HT-2 toxin uptake by BeWo cells (n = 6). C, The pH dependence of 200 nM T-2 toxin and 400 nM HT-2 toxin uptake by BeWo monolayers. The extracellular pH in the preincubation and incubation media ranged from 5.0 to 8.5. The uptake of T-2 toxin
T-2 toxin uptake by BeWo cells was dependent on a proton gradient and was carrier mediated.

The uptake of T-2 toxin but not HT-2 toxin in a sodium-depleted buffer was significantly inhibited compared with the controls, suggesting that the mechanism was sodium dependent (Fig. 5D). Inhibition studies were performed with several efflux transporter inhibitors on the effects of steady-state T-2 toxin and HT-2 toxin accumulation in BeWo cells to investigate the possible involvement of efflux transporters. The P-gp and MRP family inhibitor, CSA, and the BCRP inhibitor, Ko143, did not increase T-2 toxin and HT-2 toxin uptake (Fig. 5E), implying that the role of efflux transporters was minor. Whereas, probenecid, a non-specific organic anion transport system inhibitor, sharply decreased the uptakes of both T-2 toxin and HT-2 toxin, implying that OAT family uptake transporters were likely to be involved in the progress. The effects of various OAT and OCT inhibitors on the uptake are presented in Figure 5F. DIDS and PAH, the non-specific anion exchange inhibitors of OAT, significantly inhibited the uptake of T-2 toxin. HT-2 toxin uptake was also slightly inhibited by both OAT inhibitors. It was furthermore found that OCT inhibitors inhibited T-2 toxin uptake, whereas had no effects on the HT-2 toxin uptake. The effects of metabolic inhibitors and temperature on the uptake of T-2 toxin and HT-2 toxin were studied to determine whether this uptake required cell-dependent energy expenditure (Figs. 5G and 5H). The uptake of T-2 toxin was markedly reduced when the incubation temperature decreased from 37°C to 4°C (Fig. 5G). Sodium azide (10 mM), a respiratory chain inhibitor, dramatically inhibited the uptake of T-2 toxin and HT-2 toxin by the BeWo cells. Moreover, FCCP (50 µM), a protonophore, significantly inhibited the T-2 toxin uptake (Fig. 5H), whereas had no inhibitory effect on HT-2 toxin uptake.

**DISCUSSION**

After maternal administration of radioactive T-2 toxin, the appearance of radioactivity in the fetuses was found (Lafarge-Frayssinet et al., 1990). However, it could not identify whether T-2 toxin or its metabolites was in the fetuses. Our in vitro results indicated, for the first time, that both T-2 toxin and its major metabolite, HT-2 toxin, might be able to transport across the placenta and directly affect the fetuses. Furthermore, the present study suggested that T-2 toxin was diverse from HT-2 toxin in both transport and uptake by BeWo cells, which would offer an opportunity to further illustrate the mechanisms of both T-2 toxin and HT-2 toxin entering fetuses of human beings and animals.

The concentrations of mycotoxin used in this study were in the range from 50 to 400 nM (corresponding to 23.3–184 ng/ml). The range was consistent with the levels plausibly encountered in the human plasma after consumption of heavily contaminated food (WHO, 2000). Furthermore, to correlate the toxic effects observed in animals with acceptable levels of exposure for humans, 10% increases in mycotoxin exposure were commonly used for the inter- and intraspecies variability. The present results indicated impaired proliferation but no direct cytotoxicity after exposure to T-2 and HT-2 toxins. This indicated that in our experiments, these toxins did not alter the integrity of the placental barrier. Thus, paracellular passage might not be the main route for T-2 toxin and HT-2 toxin absorption. This was similar to the results from studies performed with deoxynivalenol and ochratoxin A on intestinal cell lines (Schrickx et al., 2006; Sergent et al., 2006). Therefore, our results suggested that the toxic effects of T-2 and HT-2 toxins were associated with increased cellular accumulation of toxins, indicating that membrane transport might be the first fundamental stage in the development of embryotoxicity and teratogenicity.

The apparent absorptive permeability was higher than 10⁻⁶ cm/s, suggesting that T-2 toxin was efficiently absorbed and the toxic effect might be related to its intracellular concentration. The favoring predominant transport in the AP-BL direction of T-2 toxin was noted, suggesting that the processes might be mediated by active transport. Additionally, T-2 toxin uptake by BeWo cells was saturable at higher concentrations, temperature dependent, and strongly inhibited by metabolic inhibitors, suggesting an energy-dependent carrier-mediated process. The results revealed that BeWo cells indeed displayed a carrier-mediated uptake of T-2 toxin across the apical membrane. Surprisingly, it was found that the transepithelial transport of T-2 toxin in the concentration range from 0 to 20 mM and HT-2 toxin by BeWo cells was measured in the presence of the indicated pH at 37°C for 3 min. Each data point represented the mean ± SD (n = 3). *p < .05 as compared with the pH 7.4 condition. D, Ionic dependence of 200 nM T-2 toxin and 400 nM HT-2 toxin apical uptake by BeWo cells. Cells were incubated at 37°C with T-2 toxin or HT-2 toxin for 3 min. NaCl in the extracellular medium was isotonicity replaced by either LiCl, choline Cl, or NaF. Mean ± SD (n = 3) were given. **p < .01 as compared with the control condition. E, Effect of inhibitors of efflux transporters on 200 nM T-2 toxin and 400 nM HT-2 toxin uptake by BeWo cell. T-2 toxin and HT-2 toxin uptake was measured at 37°C for 30 min in the presence of designated inhibitors. Cyclosporin A, Ko143, and probenecid were used at the concentrations of 10 µM, 10 µM, and 2 mM, respectively. Each point represented the mean ± SD (n = 3). **p < .01 as compared with the control condition. F, Effect of several inhibitors on T-2 toxin and HT-2 toxin uptake by BeWo cells. Uptake was measured at 37°C for 5 min in the presence of appointed inhibitors. Zidovudine (1 mM) was used as a control. The DIDS and PAH were used as inhibitors of organic anion transporters at 2 mM. TEA and cimetidine were used as an organic cation transporter inhibitor at 2 mM. Each point represents the mean ± SD (n = 3). *p < .05 as compared with the control condition, **p < .01 as compared with controls. G, Effects of incubation temperature on T-2 toxin and HT-2 toxin uptake by BeWo cells (n = 6). *p < .05 as compared with 37°C condition. H, Effects of metabolic inhibitor (NaI) and ionophore (FCCP) on the uptake of T-2 toxin and HT-2 toxin by BeWo cells. BeWo cells were pretreated with these agents. After 15-min incubation with the drugs, uptake experiments were performed. Each value represented the mean ± SD (n = 6). *p < .05 as compared with the control condition. Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; FCCP, carbonylcyanine-p-trifluoromethoxyphenylhydrazone; PAH, 4-aminohippuric acid; TEA, tetraethylammonium chloride.
was nonsaturable, implying that simple diffusion dominated the transepithelial flux. Furthermore, it was found that the inhibitors such as verapamil, MK571, Ko143, NaN₃, and 2-DOG had no effects on the BL-AP transport of T-2 toxin, suggesting no involvement of transporters in the BL-AP transport of T-2 toxin.

The uptake of T-2 toxin in BeWo cells was Na⁺ dependent and pH dependent and was inhibited by the protonophore FCCP. Because of the existences of Na⁺/H⁺ exchanger on the brush border membrane of trophoblast cells, it was proposed that the H⁺ gradient generated by the pH might be the driving force of this proton-coupled active transporter in the placenta (Ushigome et al., 2000). The present results strongly suggested that T-2 toxin uptake on the brush border membrane of BeWo cells was associated with a pH-dependent proton-coupled transport system.

The effects of various inhibitors on the uptake of T-2 toxin were first scrutinized. The present results showed that DIDS, PAH, and probenecid, well known inhibitors of the anion exchange system, significantly inhibited the uptake of T-2 toxin, indicating that T-2 toxin transported across the human trophoblast by an anion exchanger might contribute to its uptake in BeWo cells although it was unknown yet whether T-2 toxin was a substrate for anion exchange systems or not. OAT and OCT coexist in the transporting progress of aflatoxin B₁ and FB₁ entering liver and kidney (Tachampa et al., 2008). Similarly, OAT and OCT were found to play roles together in the uptake of T-2 toxin. OATs and OCTs were reported to mediate Na⁺-independent transport of their substrates (Baldwin et al., 2004). However, the present study showed that the uptake of T-2 toxin was Na⁺-dependent transport in BeWo cells, suggesting some other transporters might be involved. As reported, the organic anion-transporting polypeptide B (OATP-B) works effectively at the acidic microclimate pH (∼6), rather than at pH 7, in the absorption direction for various compounds (Kobayashi et al., 2003; Nozawa et al., 2004). In the present study, the uptake of T-2 toxin decreased when the pH was increased. Therefore, OATP-B was presumed to be involved in the uptake of T-2 toxin. Interestingly, the characteristics of the transport and uptake of folic acid and vitamin B₁ were presented (Bredeveld et al., 2007; Keating et al., 2006a,b; Takahashi et al., 2001; Yasuda et al., 2008), which were similar to that of T-2 toxin, indicating that the placental transfer of these compounds might depend on the same carrier proteins. Therefore, we concluded that folic acid and vitamin B₁ might be used as competitive inhibitors to reduce the hazard of T-2 toxin to the fetus based on the same carrier proteins. In contrast, T-2 toxin might also interact with the absorption of folic acid and vitamin B₁ and help to result in the corresponding disease caused by the shortage of the folic acid and vitamin B₁. Further research on the interaction between folic acid, vitamin B₁, and toxins in the placental transfer should be carried out.

The half-life of T-2 toxin in plasma was less than 20 min due to its rapid deacetylation to HT-2 toxin (Young et al., 2007). Some comparative data available on T-2 and HT-2 toxins indicated that they induced adverse effects with similar potency (WHO, 2001). However, no significant differences between the mean absorption and excretion rates of HT-2 toxin and no saturation in the transport were noted, which was compatible with the passive diffusion transport mechanism and was diverse from that of T-2 toxin (Fig. 6).

The mechanism of T-2-induced toxicity in pregnant rats had been reported to be related to the expression of apoptosis genes, including oxidative stress-related genes (Li et al., 2011). Therefore, the apical uptake of T-2 toxin through an active transport mechanism might be altered by an eventual concurrent apoptosis of the cells. Further study should be carried out to investigate the roles of apoptosis in the placental transfer of T-2 toxin.
Similarly with T-2 toxin, the transport of HT-2 toxin was not influenced by the inhibitors for P-gp and MRPs, indicating that T-2 and HT-2 toxins would not be a substrate of these efflux pumps. However, probenecid, DIDS, and TEA slightly inhibited the uptake of HT-2 toxin, indicating OAT carriers were involved in the uptake of HT-2 toxin in BeWo cells. Future studies are needed to determine the precise role of OATs in HT-2 toxin uptake from the maternal side.

Surprisingly, it was noted that lipid/water partition coefficient of compounds (LogP, temperature: 25°C) calculated using Advanced Chemistry Development (ACD/Labs) software, had been changed from 1.965±0.532 to 0.964±0.546 when T-2 toxin was metabolized into HT-2 toxin. When acetyl group was lost from the fourth site of T-2 toxin, the increased polarity was presumed as the important reason for their different transport and uptake characteristics. However, further research should be carried out to interpret the diverse placental transfer model between T-2 and HT-2 toxins.

In conclusion, the environmental contaminant T-2 toxin could transport across the placental cells, and the major uptake transporters such as OATs, OCTs, and OATP were involved in the process. HT-2 toxin might be transported across the epithelial cells via passive diffusion and might not accumulate in the fetal compartment. The toxicity of HT-2 toxin needs to be seriously considered due to the efficient metabolism of T-2 toxin in humans and animals. The present study provides mechanistic knowledge necessary for developing methods to prevent abnormal fetal mycotoxin-related congenital defects and therefore reduces the fetal mycotoxin contact by limiting entrance into fetal circulation or improving efflux from fetal circulation. As humans in some regions may be continuously exposed to concentrations of T-2 toxin and HT-2 toxin of health concern, the study of the potential pharmacokinetic interactions between transporter substrates and the mycotoxins should be further elucidated.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


