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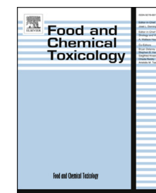

Effects of residual levels of tetracycline on the barrier functions of human intestinal epithelial cells

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ABSTRACT

Tetracyclines are frequently used in food-producing animals to treat, control, and prevent microbial diseases. Concerns are raised regarding the effects of residual levels of tetracycline, which may be present in the food supply, for emergence of drug-resistance and transfer of antibiotic-resistance gene in intestinal microbiota. In contrast, no information is available regarding the possible effects of residual tetracycline on the gastrointestinal epithelial layer barrier-disruption. This study investigates the outcome of tetracycline treatment on intestinal epithelial cells integrity. Intestinal epithelial cells (T84) were treated at concentrations of 0.015, 0.15, 1.5, 15 and 150 μg/ml for 48 h in an *in vitro* cell culture model. The permeability study revealed that 15 and 150 μg/ml of tetracycline causes barrier disruption. Whereas the altered mRNA expression of *notch-3, notch-4, claudin-2, claudin-8, claudin-10, claudin-15, gap junction alpha 8 and delta 2* and *integrin, alpha 3 and alpha L*, which are cell-integrity-related genes starts at 1.5 μg/ml tetracycline after 48 h treatment. Translocation of GFP-labeled bacteria from apical to basal comportment provides proof of concept to intestinal barrier disruption. This study is the first to evaluate whether residual concentrations of tetracycline impact epithelial cell integrity.

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1. Introduction

Tetracycline are widely used in human and veterinary medicine to treat and control infections caused by Gram-positive and Gram-negative bacteria, mycoplasma, chlamydiae, rickettsiae, and other protozoan parasites (O'Brien, 1987). Tetracycline exhibits antimicrobial activity for both extracellular and intracellular pathogens (O'Brien, 1987), and causes fewer side effects to humans and animals (Moellering, 1990; Standiford, 1990). Because tetracycline has a broad spectrum of antimicrobial activity, low toxicity, and is easily obtainable, and low-priced in the market, it has been a top choice of antimicrobial agents for use in food-producing animals since 1950 (Mellon and Benbrook, 2001).

Reports on veterinary drug product sales and distribution data reveal that during 2015, in the US alone 6,880,365 kg of tetracycline were sold, in which 71% accounts for use in food-producing animals (FDA, 2016). The FDA summary report provides detailed data showing the progressive increase in sales and distribution of antimicrobial agents approved for use in food-producing animals since 2009 (FDA, 2016). The use of antimicrobial drug products in food-producing animals may result in more than negligible residue concentrations of parent drugs or metabolites in foods of animal origin (Cerniglia and Kotarski, 2005; Cerniglia et al., 2016; Pena et al., 2010; Reyes-Herrera and Donoghue, 2008). There is concern for their potential impact on the intestinal microbiome and the development of antimicrobial-resistant bacteria in food-producing animals (Cui et al., 2005; Garofalo et al., 2007); as a result, antimicrobial new animal drugs are evaluated for their potential adverse microbiological impact on human health as part of approval process (FDA, 2003; Pena et al., 2010). Disruption of the colonization barrier and emergence of resistant bacteria in the human intestinal microbiome are a cause of concern for human

Abbreviations: IEC, Intestinal epithelial cells; TER, Transepithelial resistance; GFP, Green fluorescent labeled protein; mADI, microbiological acceptable daily intake.

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health, thus they are the endpoints identified in the human food safety evaluation of the potential impact of residues on the human intestinal microbiome (Wagner et al., 2008).

Several *in vitro* and *in vivo* studies have been conducted to understand the effect of residual tetracycline on human intestinal microbiome (Backhed et al., 2005; Cerniglia and Kotarski, 1999; Cerniglia et al., 2016; Wagner et al., 2008). In contrast, no studies are available to show if single-dose residual concentrations of tetracycline have any impact on the integrity and barrier function of the epithelial layer of the human gastrointestinal system. Pharmacokinetic studies have shown that tetracyclines are rapidly dissolved, but poorly absorbed following oral administration, leading to high concentrations in the gastrointestinal tract (JECFA, 1991). The absorption of tetracycline in the gut is influenced by the composition of ingested food. For example, calcium can interact with tetracycline and form non-absorbable chelates (Roberts, 2003). Thus, systematic studies are required to address the effects of residual levels of antimicrobial agents in the gastrointestinal system. Specifically, what concentration of tetracycline can have an impact on the integrity of the gastrointestinal epithelial layer is not known. Additionally, what experimental approaches should be used to prove if the barrier function of the intestinal epithelial layer is compromised after a single dose exposure to an antimicrobial agent needs to be determined? Furthermore, it is important to know if low concentrations of tetracycline residues in food have any effect on permeability and integrity related gene expression and phenotypic changes in intestinal epithelial cells.

Therefore, the present study examines the interaction of residual levels of tetracycline exposure to intestinal epithelial cells by using an *in vitro* cell culture model. Our hypothesis was that residual levels of tetracycline can compromise the integrity of the epithelial cell barrier function by altering gene expression that are involved in maintaining homeostasis of a healthy intestine. To test this hypothesis, *in vitro,* an intestinal epithelial cell (IEC; T84) culture model was used to determine the effects of residual levels of tetracycline on epithelial barrier function. This *in vitro* culture system is an excellent model to study the human gut epithelium, because it secretes mucin that mimics the human intestine (Donato et al., 2011; Williams et al., 2016). Additionally, polarized epithelial monolayers cultured on transwell allow measurement of changes in permeability against a wide range of xenobiotic compounds (Donato et al., 2011; Monnappa et al., 2016; Williams et al., 2016). T-84 cells expresses various claudin genes in the polarized condition, whereas some other commonly used intestinal cells (like Caco-2) cells do not express all *claudi*n genes in polarized condition (Grefner et al., 2012).

The accepted daily intake (ADI) represents an estimate of the daily intake of total drug residue in edible tissues which can be ingested over the lifetime of an individual without appreciable risk in humans (JECFA, 1991); the mADI value for tetracycline established by the Joint Expert Committee of Food Additives (JECFA) is 0–30 μg/kg bw/day and the codified ADI for the United States, is 25 μg/kg bw/day (Commission, 2009; US-FDA, 2012). Thus, the safe maximum concentration of tetracycline residues that can be ingested by an individual (standard 60 Kg body weight) per day and reaches the colon is calculated as 1.5 μg/ml. The ADI limits set by EMEA (European Agency for the Evaluation of Medicinal Products) is about 10 times lower than the JECFA and US FDA (EU-Commission-Regulation, 2010).

The Veterinary International Conference on Harmonization (VICH) guidance document identifies two microbiological endpoints of public health concern when establishing a mADI that include, 1) colonization barrier, and 2) An increase in population of resistant bacteria (Cerniglia et al., 2016). The current study was conducted to assess the effects of this residual amount on the additional endpoint, i.e. the intestinal permeability. To our knowledge, this is the first study to evaluate the effects of single low doses of tetracycline concentrations on intestinal epithelial permeability using T84 cells. Further, this study also addresses the effects of residual levels of tetracycline on the expression of permeability-related genes in intestinal epithelial cells.

2. Materials & methods

2.1. Cell culture

T84 cells (ATCC® CCL-248™), a human colorectal carcinoma cell line, were obtained from ATCC (Manassas, VA). Cells were cultured in complete growth media, which was composed of Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium supplemented with L-glutamine and HEPES (ATCC), with added 5% fetal bovine serum, penicillin/streptomycin, and fungizone. Initially, a 75 cm^2 cell culture flask was used to grow the cells until they reached 70–80% confluence. Then cells were detached using 0.25% trypsin-EDTA solution and washed twice with DMEM/F-12 medium. To wash the cells, the detached cells were collected in 15 ml (sterilized) tube then centrifuged at 500 to 600 RPM for 10 min. The media was decanted and cell pellet was suspended with antibiotic and FBS free media. This procedure was repeated once again before counting and plating for further experiment. This washing step helps to removes trypsin from cell pellet. The presence of trypsin would interfere with the cell attachment to the surface of the tissue culture plate. Cells were counted, then seeded (2.0×10^5 cells/well) into transwells and maintained in a 37 °C incubator with 5% CO_2 and 95% humidity until transepithelial resistance (TER) values were stabilized. The concentrations of tetracycline used in this study were based on the published scientific literature (Carman et al., 2005; Cerniglia and Kotarski, 1999, 2005; Cerniglia et al., 2016; Van Marwyck, 1958). There are several *in vitro* culture models and *in vivo* studies that reported tetracycline concentration that simulate exposure levels in the colon based on the ADI and safety evaluation of tetracycline residue concentrations in food (Cerniglia and Kotarski, 1999; Cerniglia et al., 2016). A dose level of 0.15 μg/ml corresponds to the United States ADI value 25 μg/kg/bw/day which considering a standard 60 kg body weight is equal to 1.5 mg of tetracycline/person/day. We used a range of concentrations above and below the ADI. Thus 1.5 μg/ml is considered as the residual concentration level for this investigation.

2.2. Transepithelial electrical resistance (TER)

The TER measurement was performed according to methods reported elsewhere (Adams et al., 1993; Bruewer et al., 2003; Donato et al., 2011; Khare et al., 2012; Williams et al., 2016; Youakim and Ahdieh, 1999). Briefly, T84 cells were seeded in the apical compartment of 6.5 mm, PFTE, collagen-coated transwell inserts (Corning, Corning, NY) at a concentration of 2×10^5 cells/well. Complete cell culture media was added to apical (0.2 ml) and basal reservoirs (0.8 ml) and cells were allowed to grow for 5–7 days to polarize (Williams et al., 2016). Then antibiotic-free medium was added to apical and basal reservoirs and TER was measured periodically using a STX electrode probe and EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL). Once the wells had reached approximately 800–1000 Ωcm^2, the medium was changed and cells were allowed to equilibrate for 3 h. Tetracycline was dissolved in ethanol (10 mg/ml) and further diluted in cell culture media for the TER studies. We conducted a preliminary experiments using two controls that include 1) media control, and 2) media containing ethanol (final concentration is 1.5%) control (150 μg/ml of tetracycline concentration contains 1.5%

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