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In vitro study of intestinal transport of fluoride using the Caco-2 cell line

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ABSTRACT

Water and food are the main sources of fluoride exposure and therefore it is necessary to study intestinal absorption in order to make a correct evaluation of the risk/benefit associated with exposure to fluoride. The present study characterizes intestinal transport of fluoride, using the Caco-2 cell line as a model of the intestinal epithelium, and evaluates the coefficients of apparent permeability and intracellular accumulation in various conditions (pH, temperature, opening of cell junctions, presence of anions).

The results indicate that fluoride is an element with moderate absorption (<70%) in both directions (absorptive and secretory). Both in absorption (apical–basolateral) and in secretion (basolateral–apical) there is transport by the paracellular pathway, which may be considered predominant. Absorption and secretion of fluoride increase at acid pH levels, possibly because of its non-ionized state at these pHs and/or because of participation of a F^-/H^+ cotransporter or a F^-/OH^- antiporter. The results also suggest transcellular participation of mechanisms involved in transport of Cl^- and of an active transport in the secretory direction. The present study extend the knowledge on the cellular transport of fluoride and provide the basis for future studies aimed at identifying potential transporters involved in human fluoride absorption.

1. Introduction

Fluorine as fluorides represents about 0.1% of the minerals present in the earth's crust (Fawell et al., 2006). It is an important component of the human organism, associated especially with calcified tissues (bones and teeth); it inhibits initiation and progression of dental caries and stimulates bone formation (Ozsvath, 2009).

Drinking water is considered the main pathway for human exposure to fluoride. The World Health Organization (WHO, 2004) recommends that fluoride concentrations in water should be less than 1.5 mg/l, although in some regions of Argentina, China, India, Kenya and Mexico this limit is greatly exceeded (Trejo-Vázquez and Bonilla-Petriocelt, 2001; Paoloni et al., 2003; Fawell et al., 2006). Food can also contribute to exposure to fluoride. Seafood, tea infusions and fluoridated salt are the products with the highest concentrations of fluoride, generally greater than 1 mg/kg (USDA, 2004; EFSA, 2005; Fawell et al., 2006). Moreover, in the child population an important daily contribution comes from the use of toothpaste (20–40 μg/kg body weight, Health Canada, 2010). The Institute of Medicine of the US National Academy of Science (IOM, 1997) proposes a reference value for dietary fluoride intake that varies according to the age group, from 0.01 mg/day in babies less than 6 months old to 4 mg/day in adults.

Because of its ability to bind to calcium and magnesium, chronic exposure to high concentrations of fluoride produces negative health effects which begin to appear as dental fluorosis and may eventually produce skeletal fluorosis (IOM, 1997; ATSDR, 2003; NRC, 2006). Additionally, studies in endemic areas have shown a reduction of cognitive capabilities in the child population (Guojian et al., 1996; Lu et al., 2000; Shivaprakash et al., 2011).

An important aspect in the evaluation of the risk associated with intake of fluoride from food is its bioavailability (quantity of the element that reaches the systemic circulation after ingestion and that is available to carry out its biological activity). The studies that have been conducted show high absorption of this element when present in water (70–90%, Cerklewski, 1997; Wang et al., 2001), although absorption may be reduced when it is present in food (Trautner and Einwag, 1989; Cerklewski, 1997). The few studies on mechanisms of intestinal transport of fluoride have been conducted with the use of various animal models (rodents, rat intestinal everted sacs, rabbit brush border membrane vesicles) and show data that are sometimes contradictory. Most of the studies indicate that intestinal absorption of fluoride is by simple passive diffusion, although Parkins et al. (1966), using different regions of rat intestinal everted sacs, showed the participation of an active component in the jejunum and ileum. It has also been suggested that fluoride may use the same transport mechanisms as chloride, and an *in vitro* study has even shown a reduction in chloride transport when fluoride is present in the medium (Dalmark, 1976; Simchowitz, 1988). Furthermore, in a study using

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rabbit intestinal vesicles, He et al. (1998) concluded that fluoride transport may be mediated by a F^-/H^+ cotransporter or a F^-/OH^- antiporter, showing a dependency on pH. The possible participation of a pH-dependent transport has been shown *in situ* on a gastric level in studies with rats (Whitford and Pashley, 1984). However, other *in vitro* studies using segments of intestine in an Ussing chamber showed that on an intestinal level absorption is mediated by transport that is not dependent on pH (Nopakun and Messer, 1990). This lack of agreement may be due, in part, to the use of different animal models. Furthermore, none of the studies that have been conducted on intestinal transport mechanisms uses a model of human intestinal epithelium.

There are various *in vitro* models for evaluating intestinal absorption in humans, although the one that is most widely used is the Caco-2 cell line. This cell line, derived from colon adenocarcinoma, differentiates spontaneously in culture, producing a monolayer of epithelial cells which shares many of the morphological and functional characteristics of mature enterocytes (Hidalgo et al., 1989). There are studies showing that in Caco-2 cells there are transporters typical of the small intestine (Maubon et al., 2007), and therefore it is considered a good model for the evaluation of intestinal transport mechanisms. This model is currently the one most commonly employed for *in vitro* study of absorption of pharmaceuticals and minerals (Ca, Fe, Zn); however, it is seldom used in the study of trace elements (Eklund et al., 2003; Laparra et al., 2005; Calatayud et al., 2012) and only one of these works refers to fluoride (Rocha et al., 2012).

The aim of the present study was to characterize the possible transport mechanisms involved in intestinal absorption of fluoride, using the Caco-2 human cell line as a model of the intestinal epithelium.

2. Materials and methods

2.1. Reagents

A fluoride standard (NaF, 1000 mg/l as fluoride, Panreac, Spain) was used for performing the experiments. For the quantification of fluoride by ion-selective electrode (ISE), standards and samples were diluted with TISAB II (total ionic strength adjustment buffer). The TISAB solution was prepared using 58 mg/ml of NaCl (Panreac), 10 mg/ml of *trans*-1,2 diaminocyclohexane-*N,N,N′,N′*-acid tetraacetic monohydrate (Fluka, Spain) and 57 μl/ml of glacial acetic acid (Panreac). The TISAB II pH was adjusted to values between 4.8 and 5.2 using 7% (m/v) of NaOH (Prolabo, Spain). Analytical-reagent grade chemicals and deionised water (18.2 MΩ cm) obtained with a Milli-Q water system (Millipore Inc., Millipore Ibérica, Madrid, Spain) were used.

2.2. Culture of Caco-2 cells

The Caco-2 cells were acquired from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK). Cell maintenance was performed in 75 cm^2 flasks using Dulbecco's Modified Eagle Medium (DMEM) with glucose (4.5 g/l) at pH 7.2. The DMEM was supplemented with 10% (v/v) of fetal bovine serum, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 100 U/ml of penicillin, 0.1 mg/ml of streptomycin and 0.0025 mg/l of amphotericin B (DMEMc). All the reagents used were acquired from PAA Laboratories GmbH (Germany).

The cells were incubated at 37 °C in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The medium was changed every 2–3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 mg/ml) and EDTA (ethylene diamine tetraacetic acid, 0.2 mg/ml) (PAA) and reseeded at a density of 5×10^4 cells/cm^2. The cells were used between passages 27 and 39.

The fluoride transport assays were performed in 6-well plates with inserts with a polyester membrane (diameter 24 mm, pore size 0.4 μm; Transwell®, Costar Corp., USA). In this system the Caco-2 cells are seeded on the porous support of the insert which separates the well into two compartments: apical (upper) and basal (lower). The cells were seeded at a density of 7.5×10^4 cells/cm^2 and they were supplemented with 1.5 ml of DMEMc in the apical chamber and 2 ml of DMEMc in the basal chamber. The cells were incubated at 37 °C, 5% CO_2 and 95% relative humidity, and the medium was changed every 2–3 days until cell differentiation took place (14–15 days post seeding).

The fluoride intracellular accumulation studies were conducted in 6-well plates. The cells were seeded at a density of 3.5×10^4 cells/cm^2, supplemented with 2 ml of DMEMc and incubated at 37 °C, 5% CO_2 and 95% relative humidity. The medium was changed every 2–3 days until cell differentiation took place.

2.3. Cell monolayer integrity

The formation and evolution of the Caco-2 monolayer was evaluated during the differentiation stage by measuring transepithelial electrical resistance (TEER) using a Millicell-ERS (Millipore Corp, Madrid, Spain). The cells were considered differentiated and suitable for the assays when the TEER values were greater than 250 Ω cm^2.

In the transport assays, monolayer stability was verified by measuring TEER at different times and determining the apparent permeability coefficient (P_{app}) of Lucifer Yellow (LY), a fluorescent compound transported mainly across cell junctions. This compound was added at a concentration of 100 μM on the apical side of the control wells and the wells treated with fluoride. Transport of LY to the basolateral side was measured with a microplate reader (PolarSTAR OPTIMA reader, BMG-Labtech, Offenburg, Germany) at an excitation/emission wavelength of 485/520 nm. The transport assays were only considered valid if, (a) the P_{app} values for LY at the end of the assay did not differ by more than 25% from the values found in the control cells and (b) the TEER values did not vary by more than 25% from those observed before the beginning of the experiment.

2.4. Cell viability assays

The effect of various concentrations of fluoride on the viability of Caco-2 cells was evaluated by using sodium resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma). The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydroresorufin, measurable by colorimetric methods. This conversion is intracellular, facilitated by mitochondrial, microsomal and cytosolic oxidoreductases (O'Brien et al., 2000).

The cells were seeded at a density of 2.5×10^4 cells/cm^2 in 24-well plates and supplemented with 1 ml of DMEMc. After differentiation took place, the cells were exposed to various concentrations of fluoride (2.5, 10, 20, 50 and 100 μg/ml prepared in DMEMc medium without fetal bovine serum; equivalent to 0.13, 0.53, 1.1, 2.6 and 5.2 mM) for 24, 48 and 72 h. After exposure, the medium was withdrawn and the culture was washed with phosphate buffered saline (PBS, PAA). Then 500 μl of resazurin solution (10 μg/ml in DMEMc without serum) was added and it was incubated for 2 h at 37 °C, 5% CO_2 and 95% relative humidity. A volume of 100 μl for each condition studied was transferred to a 96-well plate and resazurin reduction was measured colorimetrically (570 and 600 nm) using a PowerWave HT microplate scanning spectrophotometer (BioTek Instruments).

2.5. Transport and cellular accumulation assays. Calculation of apparent permeability coefficients (P_{app})

The transport assays were performed in Hanks' balanced salt solution medium with $NaCO_3$ (HBSS) (PAA) supplemented with 10 mM of HEPES (pH 7.2). The studies were conducted in the apical–basal direction (A–B) and the basal–apical direction (B–A). The standard solutions of fluoride prepared in HBSS 10 mM HEPES [2.5, 10, 20 and 50 μg/ml, equivalent to 0.13, 0.53, 1.1 and 2.6 mM] were added to the donor compartment (apical or basolateral, depending on the transport direction). At the established times (60, 120, 190 and 260 min), the contents of the acceptor compartment (basolateral or apical, depending on the transport direction) were totally removed and replaced by an equal volume of fresh medium. Fluoride was determined in aliquots of the acceptor medium removed at each time and in the donor medium removed at the end of the experiment.

The apparent permeability coefficients (P_{app}) were calculated by using the following equation:

$$P_{app} = (dC/dt)(V_r/AC_o) \quad (1)$$

where dC/dt is the flow (μg/s) determined by the linear slope of the equation that governs the variation in the concentrations of fluoride, corrected with dilution, against time, V_r is the volume of the acceptor compartment (2 ml in the A–B direction or 1.5 ml in the B–A direction), A is the surface occupied by the cell monolayer (4.67 cm^2), and C_o is the initial concentration of fluoride in the donor compartment (μg/ml).

The efflux ratio (Er) was calculated using the following equation:

$$Er = P_{app}(B{-}A)/P_{app}(A{-}B) \quad (2)$$

where P_{app}(B–A) is the apparent permeability coefficient in the basolateral–apical direction (cm/s), P_{app}(A–B) is the apparent permeability coefficient in the apical–basolateral direction (cm/s).

The cellular accumulation assays were performed in DMEMc. The differentiated cells were exposed to 20 μg/ml (1.1 mM) of fluoride for various times (2, 5, 10, 15, 30 min and 1, 2, 4, 8, 24 and 48 h). After exposure, the medium was removed, the