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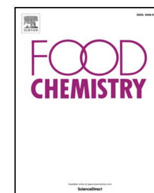

Spermine and spermidine are cytotoxic towards intestinal cell cultures, but are they a health hazard at concentrations found in foods?

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ABSTRACT

Spermine and spermidine are polyamines (PA) naturally present in all organisms, in which they have important physiological functions. However, an excess of PA has been associated with health risks. PA accumulates at quite high concentrations in some foods, but a quantitative assessment of the risk they pose has been lacking. In the present work, the cytotoxicity of spermine and spermidine was evaluated using an *in vitro* human intestinal cell model, and employing real-time cell analysis. Both spermine and spermidine showed a dose-dependent cytotoxic effect towards the cultured cells, with necrosis the mode of action of spermidine and perhaps also that of spermine. Spermine was more cytotoxic than spermidine, but for both PA the concentrations found to be toxic were above the maximum at which they have been found in food. The present results do not, therefore, support the idea that spermine or spermidine in food is harmful to healthy people.

1. Introduction

Biogenic amines (BA) are biologically active, nitrogenous organic compounds synthesised by all living organisms. They are classified as monoamines (tyramine and β-phenylethylamine), diamines (histamine, putrescine, cadaverine and tryptamine) or polyamines (PA) (spermine and spermidine) according to the number of amino groups in their chemical structure (Ladero, Linares, Perez, del Rio, Fernandez, & Alvarez, 2017). However, since the 1990s, PA have been understood as set apart from BA given their different biosynthesis and the roles they play in eukaryotic cells (Kalac, 2014). Putrescine, the precursor of spermine and spermidine, has been classified as both a BA and PA. In the present work, only spermine and spermidine are regarded as PA.

In mammalian cells, spermidine and spermine are positively charged at physiological pH, with three and four positive charges respectively. These PA are among the major cations present in cells, and are mainly found bound to polyanionic molecules such as DNA, RNA (indeed, most PA is found in the form of a PA-RNA complex), ATP and phospholipids (Igarashi, & Kashiwagi, 2000). PA are involved in the regulation of cell growth and proliferation, in controlling DNA transcription, RNA translation, protein biosynthesis, the activity of ion channels, the modulation of kinase activity, apoptosis, and in regulating the immune response (Igarashi & Kashiwagi, 2010; Larque, Sabater-Molina, & Zamora, 2007; Moinard, Cynober, & de Bandt, 2005; Pegg, 2013; Ramani, De Bandt, & Cynober, 2014). They are also clearly involved in the growth, maturation and regeneration of the intestinal cells (Kalac, 2014), possess potent antioxidant activity at physiological concentrations that prevents the damage of cell membranes and DNA (Pegg, 2013), and may play an important role in preventing food allergies in children (Dandrifosse et al., 2000).

Intracellular PA concentrations are strictly maintained by intricate multiple feedback mechanisms involved in their *de novo* biosynthesis, catabolism, and transport into and out of the cell (Igarashi & Kashiwagi, 2010; Miller-Fleming, Olin-Sandoval, Campbell & Ralser, 2015; Wallace, Fraser, & Hughes, 2003). The deregulation of PA homeostasis is associated with a number of pathological conditions such as neurological disorders, inflammation, cerebral stroke, kidney failure and cancer (Kalac, 2014; Park & Igarashi, 2013; Pegg, 2013).

In humans, the body pool of PA derives from the endogenous biosynthesis of these compounds within the cells, and from exogenous sources, i.e., from PA-producing intestinal bacteria and from the consumption of PA-rich food (Kalac, 2014; Larque et al., 2007; Ramani et al., 2014). Indeed, the diet provides larger quantities of these compounds than does intracellular biosynthesis (Bardocz et al., 1995; Kalac,

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2014). Dietary PA are quickly absorbed by the gut, presumably through an active transport process (Pegg, 2013; Poulin, Casero, & Soulet, 2012). Spermine and spermidine are found in foods of both plant and animal origin, although their content varies widely (Eliassen, Reistad, Risøen, & Rønning, 2002; Kalac, 2014). Offal (e.g., liver and kidneys) and processed fish are some of most PA-rich of all foods, but some legumes (dry and fermented soybeans, adzuki beans, cowpeas), fruits (passion fruit), shellfish (Pacific oysters), meat products and cheese (Kalac, 2014) also contain large amounts.

Despite the important physiological involvement of these amines in many cell functions, PA can pose health hazards. In laboratory animals, the administration of spermine and spermidine causes acute reductions in blood pressure, respiratory symptoms and nephrotoxicity, etc. (Pegg, 2013; Til, Falke, Prinsen, & Willems, 1997). They are also involved in carcinogenesis, tumour invasion and metastasis (Ramani et al., 2014), with cellular concentrations clearly increased in different types of cancer (e.g., colorectal and breast cancer) (Wallace & Caslake, 2001). Reduced levels of polyamine oxidase – an enzyme involved in the oxidative catabolism of spermine and spermidine – has also been reported in these cancers (Wallace & Caslake, 2001). The catabolic enzyme spermine/spermidine N^1-acetyltransferase, which reduces cell PA contents, also appears to reduce cell growth, migration and invasion in hepatocellular carcinoma and colorectal cancer (Wang et al., 2017). However, despite excess PA being associated with these potential health risks, and many foods having quite high PA concentrations, insufficient work has been done to establish legal limits in foodstuffs.

Our group has recently developed an *in vitro* model to assess the cytotoxicity of BA, individually and in combination, in human intestinal cell cultures using real-time cell analysis (RTCA) (del Rio et al., 2017; del Rio et al., submitted; Linares et al., 2016). The model was shown useful in i) determining that the dietary BA tyramine, histamine, putrescine and cadaverine are toxic towards intestinal cell cultures at concentrations found in BA-rich food, and ii) in revealing that they have different cytotoxic modes of action; while tyramine, putrescine and cadaverine cause cell necrosis, histamine induces apoptosis (del Rio et al., submitted; Linares et al., 2016). This model has also proved useful in revealing the synergistic toxicity between tyramine and histamine towards intestinal cell cultures (del Rio et al., 2017). Using this same model, the present work examines the *in vitro* toxicity of spermine and spermidine towards intestinal cell cultures. Three toxicological dose descriptors were determined for each, i.e., the IC_{50} (the concentration of PA required to achieve half of the strongest cytotoxic effect observed by RTCA), the NOAEL (the non-observed adverse effect level) and the LOAEL (the lowest observed adverse effect level). In addition, the cytotoxic mode of action of these PA was examined by analysing their capacity to induce cell necrosis or apoptosis in these intestinal cell cultures.

2. Materials and methods

2.1. Cell line and growth conditions

The *in vitro* intestinal epithelium model was established using the HT29 (ECACC 91072201) cell line. The latter was purchased from the European Collection of Cell Cultures; cells were cultured in McCoy's 5a medium as described in del Rio et al. (2017).

2.2. Real-time cell analysis

Changes in the HT29 intestinal cell cultures caused by treatment with different doses of spermine [*N*,*N*'-bis-(3-aminopropyl)-1,4-diaminobutane] (Sigma-Aldrich, Madrid, Spain) or spermidine [*N*-(3-aminopropyl)-1,4-diaminobutane] (Sigma-Aldrich) were detected using an xCelligence Real-Time Cell Analyzer (ACEA Bioscience Inc., San Diego, CA, USA), as described in del Rio et al. (2017). Briefly, stock solutions of spermine and spermidine were prepared in water and adjusted to pH 7. HT29 cells were seeded (2×10^4 cells/well) in 16-well E-Plates (ACEA Biosciences Inc.) and incubated for 20 h in a Heracell-240 Incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) at 37 °C under a 5% CO_2 atmosphere. The cell index was continuously monitored using RTCA software 1.2.1 (ACEA Biosciences Inc.). After incubation the cells were treated with different concentrations of spermine (0, 0.80, 1.20, 1.80, 2.40, 3.23, 3.50, 3.63, 4.01, 4.47, 4.49, 5.30, 6.00, 7.40, 9.80, 12.30 and 14.80 mM) or spermidine (0, 0.63, 1.25, 2.50, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70 and 86 mM). The final volume of culture medium (supplemented with either PA) in each well was 200 µl. The cell index was then monitored for another 24 h. For comparisons, the cell index was normalized to the time point just before the addition of the PA and set to 1. All experiments were conducted in at least triplicate under each set of each conditions.

The RTCA software was also used to obtain the values corresponding to the normalized cell index after 24 h of treatment with spermine or spermidine. These values were plotted against the $\log_{10}$ value of the corresponding PA concentration to construct the dose-response curves for both PA. SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA) was used to fit the non-linear regression trend lines to sigmoid dose-response (variable slope) curves. This software also provided the coefficient of determination (R^2), the Hill slope value – which indicates the steepness of the curve – and the IC_{50} values, which were calculated for spermine and spermidine at different arbitrary time points (8 h, 12 h, 18 h and 24 h of treatment).

2.3. Live cell microscopy

Flat-bottomed 96-well microplates were seeded with 2×10^4 cells/well and incubated under identical conditions to those used in the RTCA studies. After 24 h of incubation the cells were treated with concentrations of spermine (0, 2.40, 3.63, 4.47, 7.40 and 9.80 mM) or spermidine (0, 10, 25, 40, 60 and 70 mM). Images of live cells were recorded after 24 h using an inverted LumaScope-600 Series optical microscope (Etaluma, Carlsbad, CA, USA) with a 40× objective.

2.4. Cell apoptosis

The formation of apoptosis-associated DNA fragments in the cytoplasm of the spermine- and spermidine-treated cells was measured using the Cellular DNA Fragmentation ELISA Kit (Roche Applied Science, Germany) as described in del Rio et al. (2017) with some modifications (i.e., cells were exposed to PA doses similar to the IC_{50} [4.39 mM for spermine and 25.67 mM for spermidine], and above the IC_{50} [9.80 mM for spermine and 70 mM for spermidine] for 24 h). In parallel, a negative control (untreated cells) was established to determine the spontaneous release of DNA fragments, and a positive control to estimate the incorporation of BrdU into the genomic DNA (cells treated with 1% Triton-X100). DNA fragmentation in the treated samples was indicated as a percentage of the value for the positive controls.

2.5. Cell lysis assay

RTCA cell culture supernatants collected after 24 h of incubation with the corresponding dose of spermine or spermidine were tested for the presence of lactate dehydrogenase activity (LDH) using the Cytoscan Cytotoxicity Assay Kit (G Biosciences, St. Louis, MO, USA), following the manufacturer's instructions. Negative (no lysis reagent) and positive controls (with lysis reagent) were run in parallel. The percentage of cells lysed was calculated as follows: 100 × [(492 nm absorbance of PA-treated samples – absorbance of negative control) / (absorbance of positive control – absorbance of negative control)].

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