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

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Running title: Cytotoxicity of spermine and spermidine

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.

Keywords: Biogenic amines; Cytotoxicity; Food security; Spermine; Spermidine; IC_{50}; NOAEL; LOAEL

Chemical compounds studied in this article

Spermine (PubChem CID: 1103); Spermidine (PubChem CID: 1102)

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 positives 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, Peulen, El Khefif, Deloyer, Dandrifosse, & Grandfils, 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, et al., 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, 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

95 foods of both plant and animal origin, although their content varies widely
96 (Eliassen, Reistad, Risøen, & Rønning, 2002; Kalac, 2014). Offal (e.g., liver
97 and kidneys) and processed fish are some of most PA-rich of all foods, but
98 some legumes (dry and fermented soybeans, adzuki beans, cowpeas), fruits
99 (passion fruit), shellfish (Pacific oysters), meat products and cheese (Kalac,
100 2014) also contain large amounts.
101
102 Despite the important physiological involvement of these amines in many cell
103 functions, PA can pose health hazards. In laboratory animals, the
104 administration of spermine and spermidine causes acute reductions in blood
105 pressure, respiratory symptoms and nephrotoxicity, etc. (Pegg, 2013; Til,
106 Falke, Prinsen, & Willems, 1997). They are also involved in carcinogenesis,
107 tumour invasion and metastasis (Ramani, et al., 2014), with cellular
108 concentrations clearly increased in different types of cancer (e.g., colorectal
109 and breast cancer) (Wallace & Caslake, 2001). Reduced levels of polyamine
110 oxidase - an enzyme involved in the oxidative catabolism of spermine and
111 spermidine - has also been reported in these cancers (Wallace, et al., 2001).
112 The catabolic enzyme spermine/spermidine N^1-acetyltransferase, which
113 reduces cell PA contents, also appears to reduce cell growth, migration and
114 invasion in hepatocellular carcinoma and colorectal cancer (Wang, et al.,
115 2017). However, despite excess PA being associated with these potential
116 health risks, and many foods having quite high PA concentrations,
117 insufficient work has been done to establish legal limits in foodstuffs.
118

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

143 The *in vitro* intestinal epithelium model was established using the HT29
144 (ECACC 91072201) cell line. The latter was purchased from the European
145 Collection of Cell Cultures; cells were cultured in McCoy's 5a medium as
146 described in del Rio, et al. (2017).
147
148 *2.2. Real-time cell analysis*
149
150 Changes in the HT29 intestinal cell cultures caused by treatment with
151 different doses of spermine [*N,N*'-bis-(3-aminopropyl)-1,4-diaminobutane]
152 (Sigma-Aldrich, Madrid, Spain) or spermidine [*N*-(3-aminopropyl)-1,4-
153 diaminobutane] (Sigma-Aldrich) were detected using an xCelligence Real-
154 Time Cell Analyzer (ACEA Bioscience Inc., San Diego, CA, USA), as
155 described in del Rio et al. (2017). Briefly, stock solutions of spermine and
156 spermidine were prepared in water and adjusted to pH 7. HT29 cells were
157 seeded (2 x 10^4 cells/well) in 16-well E-Plates (ACEA Biosciences Inc.) and
158 incubated for 20 h in a Heracell-240 Incubator (Thermo Electron LDD GmbH,
159 Langenselbold, Germany) at 37°C under a 5% CO_2 atmosphere. The cell
160 index was continuously monitored using RTCA software 1.2.1 (ACEA
161 Biosciences Inc.). After incubation the cells were treated with different
162 concentrations of spermine (0, 0.80, 1.20, 1.80, 2.40, 3.23, 3.50, 3.63, 4.01,
163 4.47, 4.49, 5.30, 6.00, 7.40, 9.80, 12.30 and 14.80 mM) or spermidine (0,
164 0.63, 1.25, 2.50, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70 and 86 mM). The final
165 volume of culture medium (supplemented with either PA) in each well was
166 200 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 x 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 X objective.

191

192 *2.4. Cell apoptosis*

193

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

205

206 *2.5. Cell lysis assay*

207

208 RTCA cell culture supernatants collected after 24 h of incubation with the
209 corresponding dose of spermine or spermidine were tested for the presence of
210 lactate dehydrogenase activity (LDH) using the Cytoscan Cytotoxicity Assay Kit
211 (G Biosciences, St. Louis, MO, USA), following the manufacturer's instructions.
212 Negative (no lysis reagent) and positive controls (with lysis reagent) were run in
213 parallel. The percentage of cells lysed was calculated as follows: 100 X [(492

214 nm absorbance of PA-treated samples – absorbance of negative control) /
215 (absorbance of positive control – absorbance of negative control)].
216
217 *2.6. Data and statistical analysis*
218
219 The results obtained in all experiments are expressed as the means ±
220 standard deviation of at least three independent replicates. Statistical
221 treatment involved ANOVA followed by Fisher's least significant test
222 performed using SigmaPlot software. Significance was set at $p<0.05$
223 (indicated in figures with an asterisk).
224
225 **3. Results**
226
227 *3.1. Dynamic cell responses of spermine and spermidine-treated intestinal*
228 *cells*
229
230 The exposure of intestinal cells in culture to increasing concentrations of
231 spermine and spermidine caused a progressive reduction in the normalized
232 cell index as determined by RTCA (Fig. 1A and 1B respectively). These
233 results showed a dose-dependent cytotoxic effect of spermine and
234 spermidine. The cytotoxic effect of spermine seemed to be greater than that
235 of spermidine since much higher concentrations were needed to reach the
236 maximum level of cytotoxicity observable by RTCA.
237

238 Dose-response curves for spermine and spermidine were constructed using
239 the values of the normalized cell index obtained after 24 h of PA exposure
240 (Fig. 2). Both curves fitted a sigmoid curve with R^2 = 0.997 and 0.999 for
241 spermine (Fig. 2A) and spermidine (Fig. 2B) respectively. The Hill slope for
242 spermine (-5.36) was steeper than that for spermidine (-2.34), indicating that
243 similar variations in the concentration of these PA caused a greater cytotoxic
244 effect in the spermine-treated cells than in the spermidine-treated cells.
245
246 The greater cytotoxic effect of spermine compared to that of spermidine was
247 confirmed by comparing the IC_{50} values obtained from the RTCA analysis
248 after 8, 12, 18 and 24 h of exposure (Table 1). As expected, lower
249 concentrations of spermine than spermidine were needed to reach half of the
250 maximum cytotoxic effect observed by RTCA. Spermine was shown to be
251 more cytotoxic than spermidine since its IC_{50} value was lower than that of
252 spermidine at all tested times. In fact, the IC_{50} at 24 h for spermine was
253 around 5.5 times lower than that for spermidine (IC_{50} for spermine = 4.25 ±
254 0.27 mM and IC_{50} for spermidine = 23.31 ± 2.44 mM).
255
256 The NOAEL and LOAEL values obtained for spermine and spermidine (Fig.
257 2A and 2B, respectively) confirmed the higher cytotoxicity of spermine
258 (spermine: NOAEL = 2.40 mM, LOAEL = 3.23 mM; spermidine were NOAEL
259 = 5 mM, LOAEL = 10 mM). According to these values, the cytotoxicity
260 threshold for spermine was around three times lower than that for
261 spermidine.

3.2. Microscopic examination of spermine- and spermidine-treated cell cultures

The cytotoxic effect of different concentrations of spermine and spermidine towards HT29 cell cultures was observed under the microscope (Fig. 3). The morphology and number of intestinal cells in the cultures remained apparently unaltered at spermine concentrations below 3.60 mM and at spermidine concentrations below 10 mM. Above these concentrations, the cytotoxicity of both PA became visible since the cells became rounded and their numbers considerably decreased. Cellular injury became evident after the exposure to spermine and spermidine concentrations above 7.40 mM and 40 mM respectively.

3.3. Determination of the apoptotic and cytolytic (necrotic) effects of spermine and spermidine

The capacity of spermine and spermidine to induce apoptosis was determined by quantifying the intracellular DNA fragmentation they induced using the cellular DNA fragmentation assay. Both were associated with a negligible release of DNA fragments to the cytoplasm (below 0.05% DNA fragmentation) at concentrations corresponding to their IC_{50} values, and indeed even at higher concentrations (9.80 mM for spermine and 70 mM for spermidine) (data not shown). These results indicate that neither PA causes apoptosis in the HT29 cell cultures at the studied concentrations.

The release of cytosolic LDH to the culture medium directly correlates with necrotic cell death. The LDH assay was therefore used to determine the percentage of cytolysis induced in the HT29 cell cultures by the PA. Figure 4 shows that the cultures exposed to spermine experienced a dose-dependent - although quite small - release of LDH to the extracellular media (Fig. 4A). Spermine concentrations below 4.90 mM caused negligible cytolysis (0.96%), and the highest concentration tested (9.80 mM) caused only 4.53% cytolysis. Spermidine concentrations below 40 mM caused little LDH activity (<1.43%) (Fig. 4B). However, concentrations above 50 mM induced a dose-dependent increase in LDH leakage that reached its maximum (31.02% cytotoxicity) at the highest concentration tested (86 mM). These results suggest that the cytotoxic mode of action of spermine involves necrotic cell death. In the case of spermidine, the results do seem to indicate that it exerts its cytotoxic effect via necrosis, although it should be remembered that, given its lower toxicity, the concentrations used were higher than those used for spermine.

4. Discussion

As far as we know, this is the first quantitative analysis of the *in vitro* cytotoxicity of spermine and spermidine to be reported. The results show spermine and spermidine to have a dose-dependent cytotoxic effect towards intestinal cells grown in culture. Spermine was found to be more cytotoxic than spermidine, as indicated by the IC_{50}, NOAEL and LOAEL values

311 obtained. Indeed, after 24 h of treatment the IC_{50} obtained for spermine
312 showed it to be 5.5 times more cytotoxic than spermidine. Spermidine
313 appears to exert its cytotoxic effect via necrosis, as reported for tyramine
314 (Linares, et al., 2016), putrescine and cadaverine (del Rio, et al., submitted).
315 The mode of action of spermine also seemed to involve necrotic cell death,
316 although the small amount of cytolysis observed in spermine-treated cultures
317 may indicate that other mechanisms are involved.
318
319 The necrosis observed may have been the result of the action of toxic
320 compounds formed by the oxidative catabolism of spermine and spermidine,
321 in which polyamine oxidases such as the spermine oxidase (SMO) and
322 acetylpolyamine oxidase (APAO) are involved. SMO acts on spermine to
323 generate spermidine plus hydrogen peroxide (H_2O_2) and 3-aminopropanal
324 (Pegg, 2013). H_2O_2 is a toxic reactive oxygen species capable of damaging
325 DNA and other cell components, leading to cytotoxic events, senescence,
326 apoptosis, and even the development of cancer (Battaglia, DeStefano
327 Shields, Murray-Stewart, & Casero, 2014). In fact, the treatment of certain
328 tumour cell lines with polyamine analogues that induce the activity of SMO,
329 cause the release of H_2O_2, which causes cytotoxicity in these cells (Murray-
330 Stewart, Wang, Goodwin, Hacker, Meeker, & Casero, 2008). In addition, 3-
331 aminopropanal has been shown cytotoxic towards different human cell lines
332 (Wood, Khan & Moskal, 2007) and to cause the necrotic death of neurons
333 (Ivanova, et al., 2002). Moreover, this reactive aldehyde can spontaneously
334 decompose to form acrolein, a compound more toxic than other reactive

oxygen species, including H_2O_2 (Igarashi, Uemura, & Kashiwagi, 2018; Pegg, 2013). Indeed, the acrolein produced by the oxidative catabolism of spermine causes tissue damage in age-related diseases, such as kidney failure, cerebral stroke, and dementia, etc. (Igarashi, et al., 2018). H_2O_2 is also generated by the action of APAO on the acetylated forms of spermine (N^1-acetylspermine) and spermidine (N^1-acetylspermidine) (the formation of which involves catalysis via spermidine-spermine-N^1-acetyl transferase [SSAT]) (Pegg, 2013). A spermine oxidation-mediated cell death mechanism that induces necrosis rather than apoptosis has been reported in murine leukaemia cell lines (Bonneau & Poulin, 2000). Also, spermidine oxidation is reported to cause the death of pigmented retinal epithelial cells by necrosis following the disruption of cell membranes (Ohashi, Kageyama, Shinomiya, Fujita-Koyama, Hirai, Katsuta, et al., 2017).

Unlike the dietary BA histamine, which the same *in vit,* et al., 2016), spermine and spermidine did not cause apoptosis in the present cell cultures. Although the participation of PA in cell death is doubtless, controversy exists regarding their role in apoptosis (Seiler & Raul, 2005). Most reports describe PA to be protective against apoptosis, but some have indicated them to promote it (Igarashi, et al., 2010; Seiler, et al., 2005). It appears that PA depletion may prevent or activate apoptosis depending on cell type, while increased PA concentrations may cause apoptotic cell death or the malignant transformation of cells (Seiler, et al., 2005).

359 We previously reported the present *in vitro* model to be a valuable tool when
360 trying to determine the risk of toxicity after eating food containing BA such as
361 tyramine, histamine (Linares, et al., 2016), putrescine and cadaverine (del
362 Rio, et al., submitted). It was also shown useful for assessing the synergistic
363 cytotoxicity of tyramine and histamine in combination (del Rio, et al., 2017).
364 The IC_{50} for tyramine was found to be 3.20 ± 0.04 mM, for histamine 26.00 ±
365 1.20 mM, 39.76 ± 4.83 mM for putrescine, and 40.72 ± 1.98 mM for
366 cadaverine. Comparison of the IC_{50} for spermine (4.25 ± 0.27 mM) shows
367 this PA to be slightly less cytotoxic than tyramine, but around six times more
368 cytotoxic than histamine, and 9.5 times more than either putrescine or
369 cadaverine. On the contrary, spermidine (IC_{50} 23.31 ± 2.44 mM) was about
370 seven times less cytotoxic than tyramine, slightly more cytotoxic than
371 histamine, and around twice as cytotoxic as putrescine and cadaverine.
372 These data thus confirm and quantify the lesser toxicity of these PA
373 compared to tyramine - widely referred to as one the most toxic of all dietary
374 BA (along with histamine) (EFSA, 2011). While both spermine and
375 spermidine would appear to be more cytotoxic than histamine, putrescine or
376 cadaverine towards intestinal cells in culture, the concentrations at which
377 spermine and spermidine are found in foods need to be taken into account in
378 any assessment of the risk of intoxication. Foods with high spermine
379 concentrations include processed fish (up to 258 mg/kg) (Visciano, Schirone,
380 Tofalo, & Suzzi, 2012), mammalian offal such as liver (up to 249 mg/kg)
381 (Dadakova, Pelikanova, & Kalac, 2011), kidneys (up to 124 mg/kg)
382 (Dadakova, Pelikanova, & Kalac, 2012)] and even the spleen (though this is

383 rarely eaten by humans) (up to 139 mg/kg) (Fuchs, Bauer, & Paulsen, 2009),
384 some vegetables such as cow peas (up to 138 mg/kg) (Nishibori, Fujihara, &
385 Akatuki, 2007), and shellfish (up to 123 mg/kg) (Kalac, 2014)]. Spermine is
386 also found in fermented dairy products such as cheese, although at lower
387 concentrations than in the foods indicated above (up to 55 mg/kg) (Samková,
388 Dadáková, & Pelikánová, 2013). All these concentrations are much lower
389 than the lowest concentration of spermine found to be cytotoxic for the
390 intestinal cell cultures (LOAEL= 3.23 mM, equivalent to 653.56 mg/kg).
391
392 Spermidine can accumulate in foods at higher concentrations than spermine.
393 High concentrations of spermidine can be found in mammalian offal such as
394 liver (up to 390 mg/kg) (Krausová, Kalač, Křížek, & Pelikánová, 2006), and in
395 fermented soy beans (i.e., *Natto*) (up to 478 mg/kg) (Kalac, 2014), some
396 cereals (e.g., wheat) (up to 354 mg/kg) (Nishimura, Shiina, Kashiwagi, &
397 Igarashi, 2006), processed fish (up to 258 mg/kg) (Visciano, Schirone,
398 Tofalo, & Suzzi, 2012), and mushrooms (up to 158 mg/kg) (Nishimura, et al.,
399 2006). Additionally, spermidine is found in cheese, although at lower
400 concentrations than in the previously mentioned foods (up to 73 mg/kg)
401 (Spizzirri, Restuccia, Curcio, Parisi, Iemma, & Picci, 2013). Thus, the
402 concentrations of spermidine found in food are much lower than that which
403 caused cytotoxic effects in the intestinal cell cultures (LOAEL = 10 mM,
404 equivalent to 1452.50 mg/kg). The differences are, in fact, greater even than
405 for spermine.
406

407 The amounts for spermine and spermidine found to be cytotoxic in this work
408 (653.56 mg/kg and 1452.50 mg/kg respectively) are thus far above the
409 highest concentrations found in foods. Despite their *in vitro* cytotoxicity
410 towards intestinal cells in culture, the present results do not support the idea
411 that the intake of PA-rich food is harmful for healthy people. In addition, the
412 cytotoxicity that ingested spermine and spermidine might have *in vivo* may be
413 even lower than that assessed *in vitro* since cell culture media are
414 supplemented with foetal bovine serum. The latter contains a serum amine
415 oxidase that oxidizes spermine and spermidine to generate ammonia, H_2O_2
416 and aminoaldehydes [*N'*-(4-aminobutyl)-aminopropionaldehyde and *N,N'*-
417 bis(3-propionaldehyde)-1,4-butanediamine, respectively] that finally turn into
418 the cytotoxic acrolein (Pegg, 2013). Any toxic compounds formed due to the
419 oxidation of spermine and spermidine by serum amino oxidase present in the
420 culture media could, therefore, be partially responsible for the cytotoxicity of
421 these PA observed *in vitro*.
422
423 It is, however, important to note that concentrations below the toxicity
424 thresholds established in this work might be dangerous for people with
425 cancer or chronic kidney failure, etc. The transport of extracellular PA is
426 dramatically increased upon the oncogenic transformation of cells (Poulin, et
427 al., 2012) and a high PA uptake might increase PA availability and accelerate
428 tumour growth. Certainly, a prospective observational study on hormone-
429 refractory prostate cancer shows that the reduction of dietary PA and
430 putrescine uptake may increase patient survival and improve quality of life

(Cipolla, Havouis, & Moulinoux, 2010). In fact, patients with cancer are advised not to consume PA-rich foods (Kalac, 2014; Larque et al., 2007).

Patients with chronic kidney failure show high plasma polyamine oxidase activity, which leads to increased spermine and spermidine catabolism and the accumulation of toxic acrolein (Igarashi, Ueda, Yoshida, & Kashiwagi, 2006). In such patients, a high PA might therefore have adverse effects. In addition, under acidic conditions, spermidine reacts with nitrous acid preservatives in food, forming the nitrosamines N-nitrosopiperidine and N-nitrosodimethylamine, both of which are possible carcinogens (Drabik-Markiewicz, Dejaegher, De Mey, Kowalska, Paelinck, & Vander Heyden, 2011).

In summary, this work assesses the cytotoxicity of the dietary PA spermine and spermidine using an *in vitro* model of the human intestinal epithelium previously validated for testing that of BA tyramine, histamine (del Rio, et al., 2017; Linares, et al., 2016), putrescine and cadaverine (del Rio, et al., submitted). Both spermine and spermidine showed dose-dependent cytotoxicity towards the cultured cells. The toxicity threshold for spermine was three times lower than that for spermidine (LOAEL values for spermine and spermidine 3.23 mM and 10 mM respectively). The cytotoxic mode of action of spermidine, and perhaps of spermine, was through the induction of necrosis rather than apoptosis. Although the present results highlight the cytotoxicity of spermine and spermidine *in vitro*, toxic concentrations have

455 never been found (as far as we know) in food. Accordingly, the present work
456 does not support the idea that the intake of PA-rich food is harmful for
457 healthy people.
458
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460
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464
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590

591 **7. Figure legends**

592

593 **Figure 1.** Dynamic monitoring of changes in the normalized cell index of
594 HT29 cells treated with spermine and spermidine as determined by RTCA.
595 Cells were exposed to the indicated concentrations of spermine (A),
596 spermidine (B) or control medium (0 mM). The point of administration of
597 spermine or spermidine is shown by vertical arrows on the graph. The data

598 refer to a representative experiment; vertical bars represent standard
599 deviations.
600
601 **Figure 2.** Sigmoid dose-response curves for the effect of spermine and
602 spermidine on HT29 intestinal cells. Cell cultures were treated with different
603 spermine (A) or spermidine (B) concentrations for 24 h. Data represent the
604 means ± standard deviations of at least three replicates. An asterisk
605 indicates the first significant difference with respect to the minimal dose of PA
606 assayed (0.80 and 0.63 mM for spermine and spermidine respectively) and
607 represents the LOAEL concentration (*p<0.05). Numeric values for IC_{50},
608 NOAEL, LOAEL, Hill slope and R^2 are also indicated.
609
610 **Figure 3.** Live cell imaging of HT29 cells treated with either (A) spermine (0,
611 2.40, 3.63, 4.47, 7.40 and 9.80 mM) or (B) spermidine (0, 10, 25, 40, 60 and
612 70 mM) for 24 h. Images of live cells were recorded using an inverted optical
613 microscope (magnification 40 X).
614
615 **Figure 4.** Cytolytic effect of spermine (A) and spermidine (B) on intestinal
616 cells. RTCA cell culture supernatants were collected after 24 h of incubation
617 with the corresponding concentrations of spermine or spermidine. Necrosis
618 was measured in supernatants of PA-treated RTCA cultures by measuring
619 the lactate dehydrogenase activity (LDH). Data represent the means of at
620 least three replicates; vertical bars represent standard deviations.
621

Table 1. IC_{50} values (mean ± standard deviation) for spermine and spermidine after exposure of HT29 intestinal cells for different times.

Time (h)	Spermine (IC_{50})	Spermidine (IC_{50})
8	10.60 ± 2.46	57.14 ± 12.19
12	7.49 ± 1.91	49.07 ± 22.02
18	5.35 ± 0.63	33.72 ± 12.58
24	4.25 ± 0.27	23.31 ± 2.44

Values are the mean ± standard deviation in mM

636

637

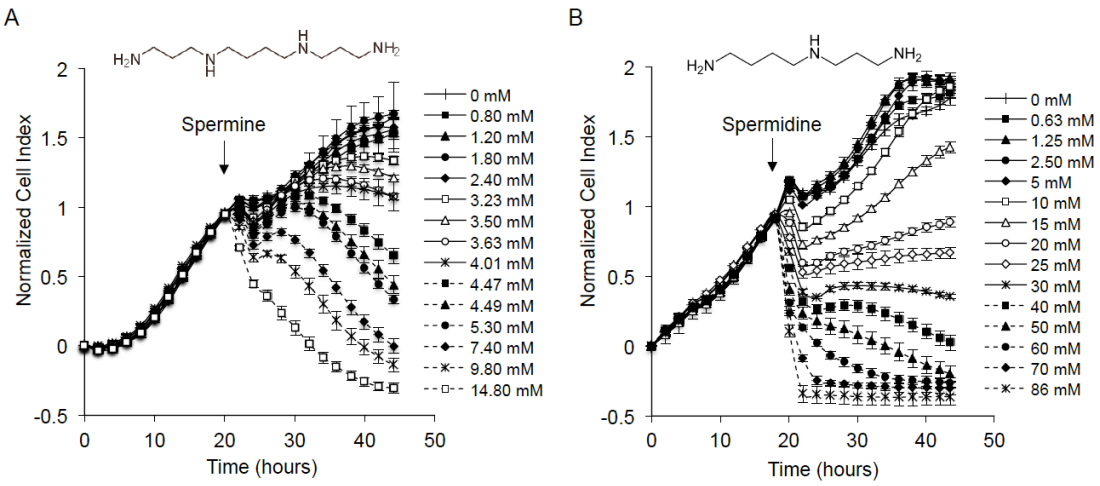


Figure 1

638

639

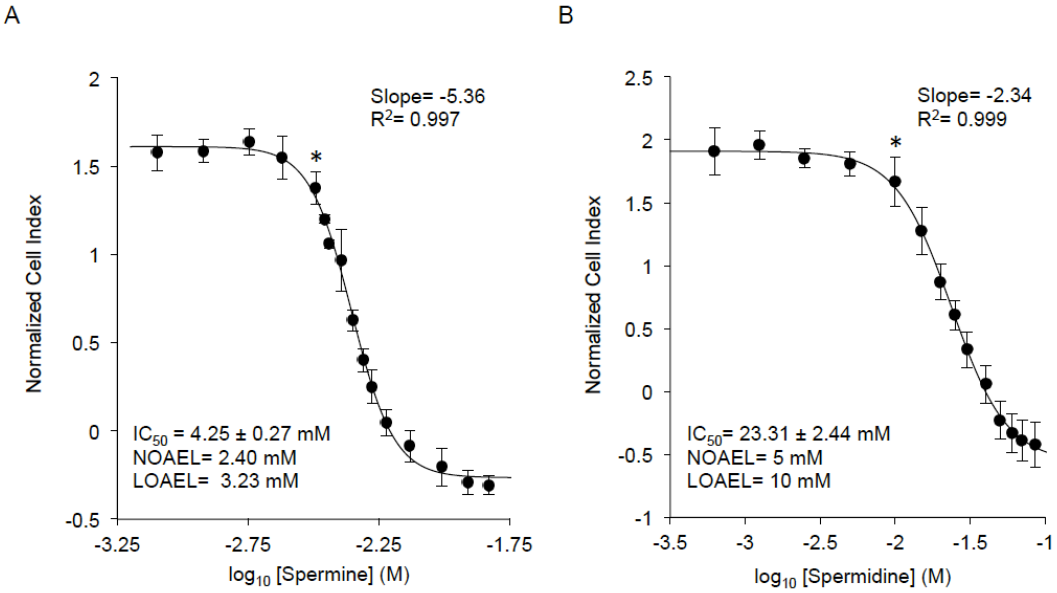
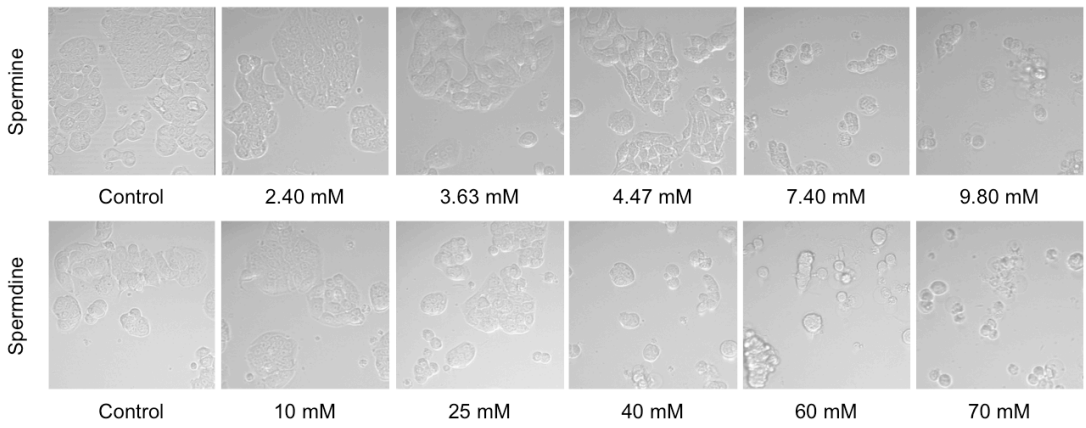


Figure 2


Spermine
Control
2.40 mM
3.63 mM
4.47 mM
7.40 mM
9.80 mM
Spermdine
Control
10 mM
25 mM
40 mM
60 mM
70 mM

640 Figure 3

641

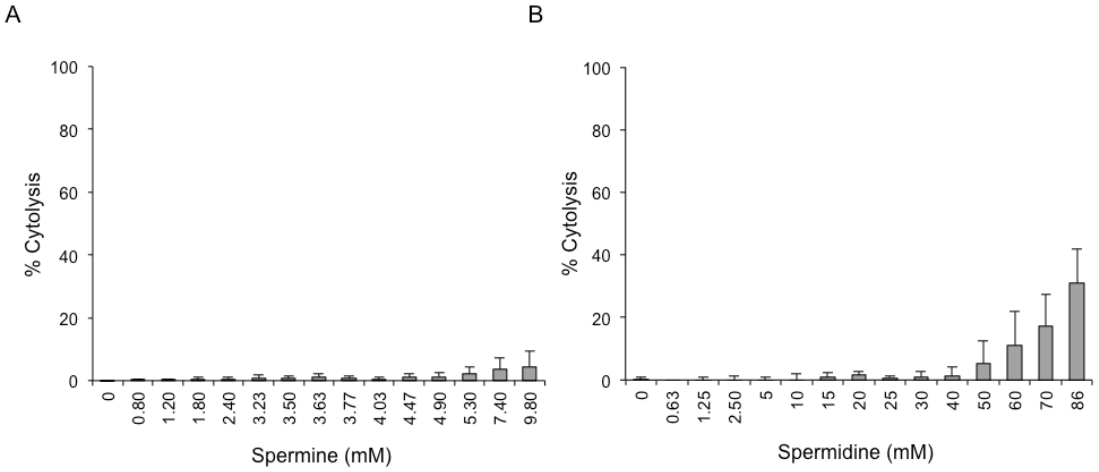

A
B
% Cytolysis
100
80
60
40
20
0
0
0.80
1.20
1.80
2.40
3.23
3.50
3.63
3.77
4.03
4.47
4.90
5.30
7.40
9.80
Spermine (mM)
% Cytolysis
0
0.63
1.25
2.50
5
10
15
20
25
30
40
50
60
70
86
Spermidine (mM)

Figure 4