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# Single and combined effects of regulated and emerging mycotoxins on viability and mitochondrial function of SH-SY5Y cells

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ABSTRACT

Co-occurrence of emerging and regulated mycotoxins in contaminated samples has been widely documented, but studies about their combined toxicity are scarce. In this report, the regulated mycotoxins deoxynivalenol, fumonisin B1 and zearalenone, and the emerging ones enniatin A, enniatin B and beauvericin were tested in SH-SY5Y human neuroblastoma cells. Their individual and binary combined effects on cell viability and mitochondrial function were evaluated. The results with individual mycotoxins revealed that deoxynivalenol and emerging mycotoxins were the most damaging to neuronal cells, presenting $IC_{50}$ values between 0.35 and 2.4 μM. Interestingly, non-regulated mycotoxins triggered apoptosis by affecting to mitochondrial membrane potential. However, when regulated and non-regulated mycotoxins were binary mixed, antagonistic effects were found in all cases. Finally, cow feed and milk extracts were analysed by UHPLC-MS/MS, detecting the presence of several mycotoxins included in this study. These extracts were tested in neuroblastoma cells, and damaging effects on cell viability were found. Although binary combinations of mycotoxins produced antagonistic effects, their mixture in natural matrixes induces greater effects than expected. Therefore, it would be interesting to explore the matrix influence on mycotoxin toxicity, and to continue studying the neurotoxic mechanism of action of emerging mycotoxins, as they could be a health hazard.

## 1. Introduction

Mycotoxins are secondary metabolites from different fungi species, highlighting those belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium*. Mycotoxins are usually present in cereals such as corn or rye, as well as in other types of food such as milk or even fish. They are physically and thermodynamically stable substances, which resist the processes of milling and drying, as well as cooking and baking, and their ingestion causes mycotoxicosis in animals and people (Bertero et al., 2020; Sainz et al., 2018).

According to the European Food Safety Authority (EFSA), mycotoxins can be divided into two large groups in relation to the severity of their effects: regulated mycotoxins and unregulated or emerging mycotoxins. Regulated mycotoxins comprise those which are a public health problem due to their high toxicity at known concentrations and, therefore, maximum limits in food have been established (Commission Regulation (EC) No 1881/2006). This group includes fumonisins, zearalenone (ZEA), aflatoxins, ochratoxin A, patulin and trichothecenes. The latter is subdivided into two sub-groups: type A, where the highly toxic T2 toxin is found, and type B, where deoxynivalenol (DON) stands out. Unregulated mycotoxins are those which are not considered a problem for public health. Currently, there are not much data about these toxins, so it cannot be stated if they are safe for animals and humans (EFSA, 2012, 2013, 2014). These mycotoxins include enniatins (ENNs),

*Abbreviations:* ATCC, American Type Culture Collection; BBB, blood brain barrier; BEA, beauvericin; CI, confidence interval; ΔΨm, mitochondrial membrane potential; DON, deoxynivalenol; EFSA, European Food Safety Authority; ENN, enniatin; FB1, fumonisin: B1; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NA, not applicable; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; Rot, rotenone; STS, staurosporine; TMRM, tetramethylrhodamine methyl ester; UHPLC-MS/MS, ultra-high liquid chromatography tandem mass spectrometry; ZEA, zearalenone.

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beauvericin (BEA), sterigmatocystine, phomopsins and *Alternaria* toxins.

Normally, food is not contaminated by a single type of mycotoxin (Arroyo-Manzanares et al., 2019). A cereal such as maize can be infested by several species of *Fusarium*, *Aspergillus* or *Penicillium* at the same time, which produce different types of mycotoxins that jointly contaminate the cereal. Studies about joint toxicity of these compounds, whose concentrations vary greatly between samples, are scarce (Agahi et al., 2020a; Jia et al., 2020; Juan-García et al., 2020). Co-occurrence of regulated and emerging mycotoxins has been previously detected in studies carried out on samples from NW Iberian Peninsula by our group and other authors (Dagnac et al., 2016; González-Jartín et al., 2020). In these works, it was outstanding the presence of the regulated mycotoxins DON, fumonisin B1 (FB1) and ZEA, while ENN A, ENN B and BEA were the prevalent emerging mycotoxins.

DON is a type B trichothecene, commonly known as vomitoxin, and produced by *Fusarium* spp. which causes a picture of vomiting, anorexia and fever whose origin can be central or peripheral. Oral ingestion of DON activates POMC neurons and the release of nesphatin-1, which are closely related to the reduction of food intake. In addition, it has cytotoxic effects on fast-growing cells such as those of the intestinal epithelium. This cytotoxicity has been associated with the ability of DON to produce ROS and lipid peroxidation (Bonnet et al., 2012; Zhang et al., 2020). FB1 causes equine leukoencephalomalacia and to be involved in the development of neural tube defects in children. To date, the pathway by which this toxin produces neurotoxicity is mostly undisclosed, but it is known that FB1 blocks a key enzyme in the metabolism of sphingolipids, altering the membrane functions (Domijan, 2012; Khan et al., 2018). Regarding ZEA, it is produced by the *Fusarium* genus and has a great oestrogenic activity due to its structure. Its toxicity is not only due to oestrogenic effects, other mechanisms such as oxidative stress are also involved (Tatay et al., 2017).

With respect to emerging mycotoxins, ENN B is the most frequent in processed and unprocessed grains in Europe and, therefore, it is the most studied one. It has antibacterial, insecticidal, and antifungal properties. At cellular level, ENN B produces mitochondrial toxicity due to its ionophoric properties, as the rest of the toxins of the enniatin family (Prosperini et al., 2017). BEA is structurally related to the enniatin family. It also acts as an ionophore, causing mitochondrial dysfunction, and has antimicrobial, entomopathogenic and antitumor activities (Bertero et al., 2020).

Although the mechanism of action of these toxins is not fully understood, they can cross the blood brain barrier (BBB) and cause neurotoxic effects (Bonnet et al., 2012; Osuchowski et al., 2005b; Taevernier et al., 2016). Their neurotoxicity could be related to their effects on mitochondria, as many of them are known to cause mitochondrial dysfunction by producing oxidative stress and inducing the formation of reactive oxygen species (ROS). Neurons are very susceptible to oxidative stress, as they consume large amounts of oxygen through oxidative phosphorylation. On the other hand, there are previous studies which conclude that both regulated and emerging mycotoxins induce apoptotic cell death (Bensassi et al., 2012; Prosperini et al., 2013). ROS can activate apoptotic cell death through the intrinsic pathway, also named mitochondrial pathway because the initiating signals of this mechanism are triggered by mitochondria (Walia et al., 2021). Therefore, mitochondrial dysfunction could be closely related with the cell death produced by mycotoxins. In fact, some studies suggest that DON can induce apoptosis through the opening of the mitochondrial transition pore (Bensassi et al., 2012).

In this study, the effects originated by the regulated and emerging mycotoxins most frequently found in contaminated samples of the NW Iberian Peninsula were analysed in SH-SY5Y human neuroblastoma cells. DON, FB1, ZEA, ENN A, ENN B and BEA were tested individually and in binary combinations to elucidate their cytotoxicity and the effects on mitochondrial function, as well as their type of interaction.

## 2. Materials and methods

### 2.1. Chemicals and solutions

Tetramethylrhodamine methyl ester (TMRM), CyQUANT™ lactate dehydrogenase (LDH) Cytotoxicity Assay Kit, Dulbecco's Modified Eagle Medium: F-12 nutrient Mix (DMEM/F-12), trypsin/EDTA (0.05%), glutamax and penicillin-streptomycin (10,000 U/mL) were bought in Thermo Fisher Scientific (Madrid, Spain). Durapore membrane centrifugal filters (0.22 μm pore size) were purchased from Merck Millipore (Billerica, USA). DON and FB1 (purity >98%) were obtained from Romer Labs (Getzersdorf, Austria). ZEA, ENN A, ENN B and BEA (purity >96%), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), saponin (SAP), staurosporine (STS), Annexin V-FITC Apoptosis Detection Kit, and another chemical reagent grade were purchased from Sigma-Aldrich (Madrid, Spain).

The composition of Locke's buffer was: 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$ 1.3 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES. Stock solutions of mycotoxins were prepared in dimethylsulfoxide (DMSO).

### 2.2. Cow feed and milk extracts and analysis

A cow feed sample was analysed according to a method previously validated for the multidetection of 22 mycotoxins in this matrix (González-Jartín et al., 2020).

For cellular assays, an aliquot of 9 mL of the extract was filtered (0.22 μm), dried in a centrifugal evaporator, and reconstituted in 0.45 mL of DMSO, obtaining a 20 times concentrated sample.

Two commercial milk samples were analysed according to a method previously validated for the multidetection of 40 mycotoxins in these matrixes (González-Jartín et al., 2021).

For cellular assays, an aliquot of 1 mL of each extract was filtered (0.22 μm), dried in a centrifugal evaporator, and reconstituted in 0.05 mL of DMSO, obtaining a 20 times concentrated sample.

### 2.3. Cell culture and treatment

SH-SY5Y human neuroblastoma cell line was purchased from American Type Culture Collection (ATCC), number CRL2266. Cells were cultured in DMEM/F-12 enriched with 10% fetal bovine serum, 1% glutamax and 10,000 U/mL penicillin-streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$ and dissociated once a week using 0.05% trypsin/EDTA.

SH-SY5Y cells were cultured in 96-well plates at concentration of $5 \times 10^4$ cells/well for all the assays performed, except for flow cytometry analysis.

Mycotoxin standards were dissolved in DMSO and serial dilutions were performed in culture medium. In all cases, DMSO levels in cells were kept below 0.5%. For individual mycotoxin assays, cells were treated at concentrations ranging from 0.1 to 30 μM. In the case of binary combinations, the ratio among regulated and unregulated mycotoxins was selected based on the most frequent proportions found in contaminated samples of the NW Iberian Peninsula (González-Jartín et al., 2020). Cells were treated for 24 h with the following mixtures: DON and FB1 were combined at 5:1 ratio with ENN A, ENN B and BEA, whilst ZEA was combined with unregulated mycotoxins at 2:1 ratio. Combined concentration gradients were selected based on the results obtained with single mycotoxins, in order to include half maximal inhibitory concentration ($IC_{50}$) values in the concentration range analysed.

With regard to the concentrated cow feed and milk extracts, SH-SY5Y cells were treated with 1:500, 1:1000, 1:5000 and 1:10000 dilutions for 24 h. In both cases, a blank sample (uncontaminated matrix) was used as control.

To compare the cow feed extract effects with commercial

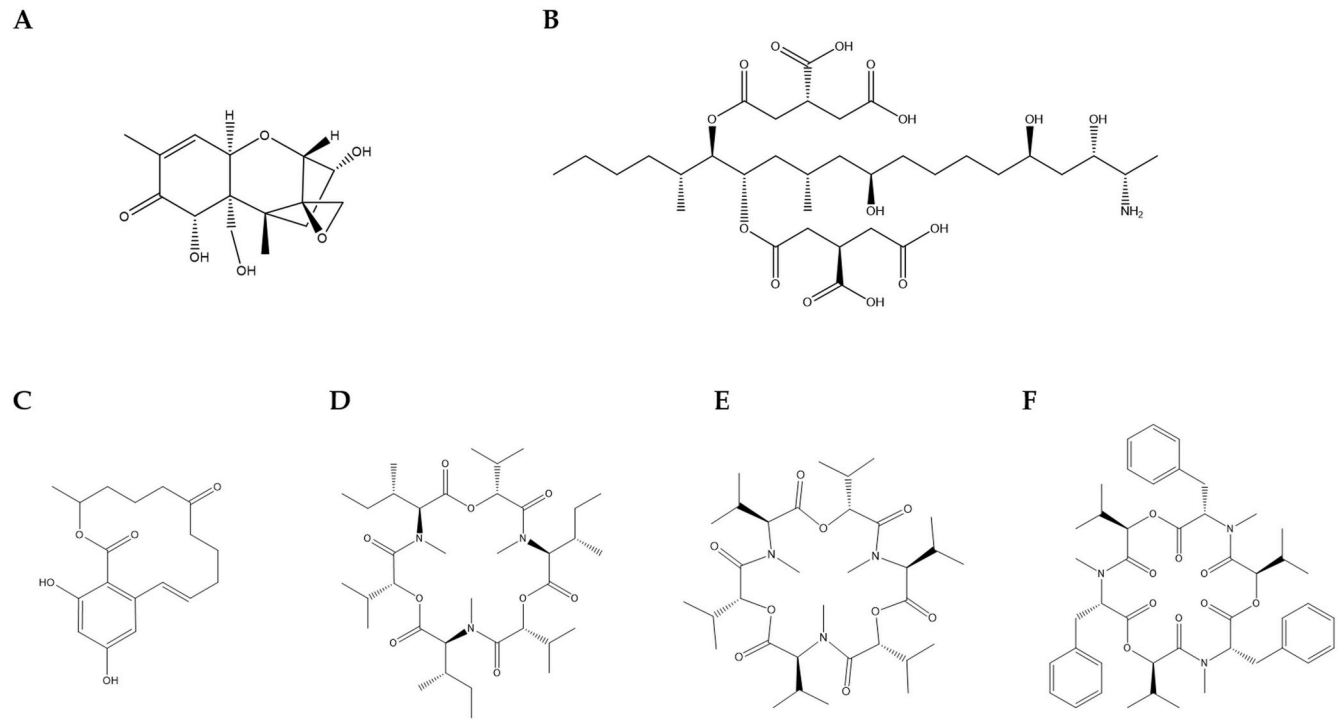


**Fig. 1. Chemical structures of regulated and emerging mycotoxins.** (A) DON, (B) FB1, (C) ZEA, (D) ENN A, (E) ENN B, (F) BEA.

mycotoxins, a commercial mycotoxin mixture was also added to the cells for 24 h. The concentrations selected for this combination were based on the results obtained from the contaminated extract analysis.

All the experiments were performed in triplicate, at least three independent times.

### 2.4. Cell viability assay

The effect of single mycotoxins, binary combinations and the concentrated extract on cell viability was analysed with MTT assay as previously described (Alvariño et al., 2017). SAP from *Quillaja bark* at 1 mg/mL was used as cell death control. Cells were treated with individual mycotoxins for 6, 24 and 48 h, whilst binary combinations and extract treatments were performed for 24 h. After treatment, cells were washed three times with Locke's solution and 200 μL of MTT (500 μg/mL) were added to each well. The plate was incubated for 1 h at 37 °C and 300 rpm in an orbital shaker. Then, MTT was removed, and 5% sodium dodecyl sulphate was added to originate cell lysis. The absorbance produced by the tetrazolium salt was read at 595 nm in a spectrophotometer plate reader.

### 2.5. Cytotoxicity assay

CyQUANT™ LDH Cytotoxicity Assay Kit test was used to analyse cell cytotoxicity, following manufacturer's instructions. Cells were cultured in 96-well plates and treated as previously described. Individual mycotoxins were added for 6 and 24 h, whereas binary mixtures and extract treatments were carried out for 24 h. SAP from *Quillaja bark* (1 mg/mL) was used as cell death control. After treatment, 50 μL of medium were transferred to a 96-well flat-bottom plate and LDH release was evaluated. Absorbance was read at 490 and 680 nm in a plate reader and the 680 nm absorbance value (background signal) was subtracted from the 490 nm absorbance value to determine LDH activity.

### 2.6. Mitochondrial membrane potential measurement

Mitochondrial membrane potential ($\Delta\Psi_m$) was analysed with TMRM dye, which is a fluorescent lipophilic cationic probe that accumulates within mitochondria in inverse proportion to $\Delta\Psi_m$. For this assay, cells were treated with single mycotoxins for 6 and 24 h, and with binary combinations for 24 h. Then, cells were washed twice with Locke's solution and 200 μL of 20 μM TMRM were added to each well. The plate was incubated for 30 min at 37 °C and 300 rpm and cells were solubilised with $H_2O$ and DMSO (1:1). Fluorescence was read at 535 nm excitation and 590 nm emission in a plate reader (Bornancin et al., 2019). Rotenone (Rot) at 1 μM was used as positive control.

### 2.7. Flow cytometry analysis

Annexin V-FITC Apoptosis Detection Kit was used to determine the type of cell death produced by mycotoxins, following manufacturer's instructions. SH-SY5Y cells were seeded in 12-well plates at $5 \times 10^5$ cells per well and allowed to grow for 24 h. Then, cells were incubated with individual mycotoxins for 24 h. STS at 0.1 μM was used as positive control (Alvariño et al., 2020). After treatment, cells were washed with phosphate buffered saline (PBS) and resuspended in Annexin-binding buffer containing Annexin V-FITC and Propidium Iodide (PI). Next, cells were incubated at room temperature for 15 min, resuspended in commercial PBS (Thermo Fisher Scientific), filtered and maintained on ice. The fluorescence was determined by flow cytometry using the ImageStreamMKII instrument (Amnis Corporation, Luminex Corp, Austin, TX, USA). The fluorescence of 10,000 events was acquired and analysed with IDEAS Application 6.0 software (Amnis Corporation, Luminex Corp).

### 2.8. Statistical analysis

Data are expressed as mean ± SEM of three independent experiments. Statistical differences were evaluated by Student's t-test. p values $\leq 0.05$ were considered statistically significant. $IC_{50}$ and half maximal effective concentration ($EC_{50}$) values were determined by fitting the data with a log (inhibitor) *vs* response model or a log (agonist) *vs* response model, respectively, with GraphPad Prism 8 software.

The combination index was calculated with Chou & Talalay equation

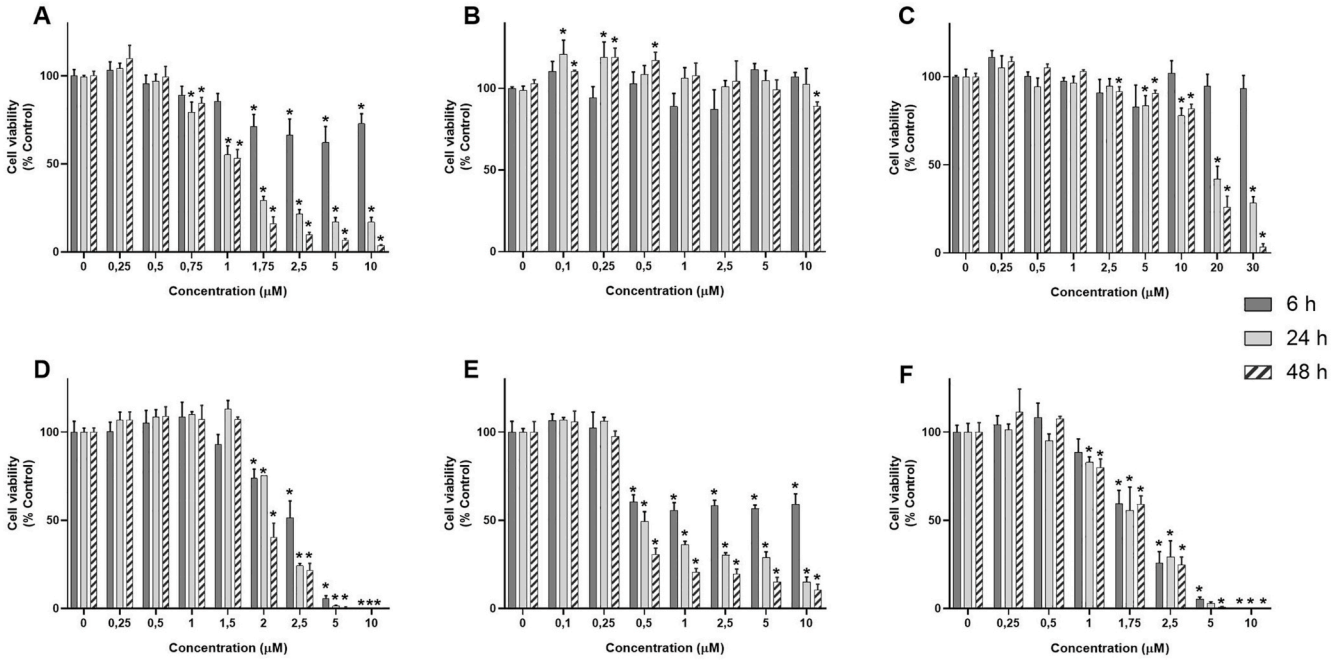


**Fig. 2. Effect of selected mycotoxins on cell viability of SH-SY5Y cells**. Cells were incubated with mycotoxins for 6, 24 and 48 h and their effect on cell viability was assessed by MTT test. (A) DON, (B) FB1, (C) ZEA, (D) ENN A, (E) ENN B, (F) BEA. Data are mean ± SEM of three independent replicates expressed as percentage of untreated control cells. Statistical differences determined by Student's *t*-test. *p ≤ 0.05, significantly different from the control.

(Chou, 2010):

$$COMBINATION\ INDEX = \frac{D1}{(DX)1} + \frac{D2}{(DX)2}$$

where (DX)1 and (DX)2 are the $IC_{50}$ values of each mycotoxin alone, and D1 and D2 are the $IC_{50}$ values of each mycotoxin in binary combination. According to Chou & Talalay method, synergism, additivity, and antagonism were considered when the combination index was <1, =1 and >1, respectively.

## 3. Results

### 3.1. Evaluation of single mycotoxin effects on cell viability and cytotoxicity

At first, MTT assay was used to analyse the effect of regulated and emerging toxins on cell viability of human neuroblastoma SH-SY5Y cells. Cells were treated with DON, FB1, ZEA, ENN A, ENN B and BEA (Fig. 1) at concentrations ranging from 0.1 to 30 μM for 6, 24 and 48 h to compare the effect produced at different incubation times.

DON did not totally produce cell death after 6 h of treatment, it only produced a slight decrease on cell viability (about 30%) at concentrations between 1.75 and 10 μM. This mycotoxin had a similar effect after 24 and 48 h of incubation, producing a significant reduction on cell viability, in a dose-dependent manner, at concentrations over 0.75 μM (Fig. 2 A). FB1 only reduced cell viability at the highest concentration after 48 h of incubation and it seemed to stimulate cell proliferation at the lowest doses used after 24 and 48 h (Fig. 2 B). The last of the regulated mycotoxins tested, ZEA, did not produce any cell damage at 6 h of incubation, but it decreased cell viability at concentrations over 20 μM at 24 and 48 h, producing a reduction of more than 50% compared to control cells (Fig. 2 C). With regard to emerging mycotoxins, treatment with ENN A for 6 h produced a reduction in cell viability of 41.0 ± 8.5% and 94.3 ± 1.6% at 2.5 μM and 5 μM, respectively, and a complete inhibition at 10 μM. At 24 and 48 h of incubation, the decrease on cell viability at 2.5 and 5 μM was higher than 74.5%, and a total reduction was found again at the highest dose tested (Fig. 2 D). In the case of ENN B, the effects observed at 6, 24 and 48 h were more pronounced than for ENN A, resulting in significant cell damage at concentrations over 0.5 μM, but the mycotoxin did not produce a total inhibition of cell viability at the highest dose (Fig. 2 E). Finally, BEA showed similar effects that ENN A, decreasing cell viability more than a 50% at concentrations above 1.75 μM at all the incubation times (Fig. 2 F).

**Table 1**
**$IC_{50}$ values of mycotoxins in MTT assay.** DON: deoxynivalenol, FB1: fumonisin B1, ZEA: zearalenone, ENN A: enniatin A, ENN B: enniatin B; BEA: beauvericin, CI: 95% confidence interval, NA: Not applicable.

| TOXIN | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | 6 h | 24 h | 48 h |
| DON | NA | 0.94 (CI:0.84–1.0)<br>$R^2$: 0.96 | 0.97 (CI: 0.88–1.1)<br>$R^2$: 0.97 |
| FB1 | NA | NA | NA |
| ZEA | NA | 17.4 (CI: 15.1–19.8)<br>$R^2$: 0.94 | 16.5(CI: 14.3–21.2)<br>$R^2$: 0.93 |
| ENN A | 2.4 (CI: 2.2–2.8)<br>$R^2$: 0.93 | 2.25 (CI: 2.2–2.3)<br>$R^2$: 0.98 | 2.0 (CI: 1.8–2.1)<br>$R^2$: 0.96 |
| ENN B | NA | 0.43 (CI: 0.37–0.48)<br>$R^2$: 0.95 | 0.35 (CI: 0.31–0.41)<br>$R^2$: 0.96 |
| BEA | 1.9 (CI: 1.6–2.3)<br>$R^2$: 0.96 | 1.7 (CI: 1.3–2.1)<br>$R^2$: 0.95 | 1.5 (CI: 1.3–1.8)<br>$R^2$: 0.96 |

With the data obtained, $IC_{50}$ values were calculated for each toxin by fitting the data with a log (inhibitor) *vs* response model (Table 1, Figure S1). At 6 h, only ENN A and BEA produced a complete reduction of cell viability, so $IC_{50}$ values were calculated for these emerging toxins. Both presented similar values, 2.4 μM (CI: 2.2–2.8 μM) and 1.9 μM (CI: 1.6–2.3 μM), respectively. Regarding longer incubation times (24 and 48 h), $IC_{50}$ values were calculated for all the mycotoxins tested, with the exception of FB1, which did not alter the cell function in this cell line. At 24 h, DON and ENN B turned to be the most damaging toxins, with $IC_{50}$ of 0.94 and 0.43 μM, respectively. Otherwise, ZEA presented the highest $IC_{50}$ (17.4 μM). The same trend was observed at 48 h, with DON and emerging mycotoxins presenting $IC_{50}$ values in the low micromolar range, whilst ZEA was again the least harmful toxin. In general,

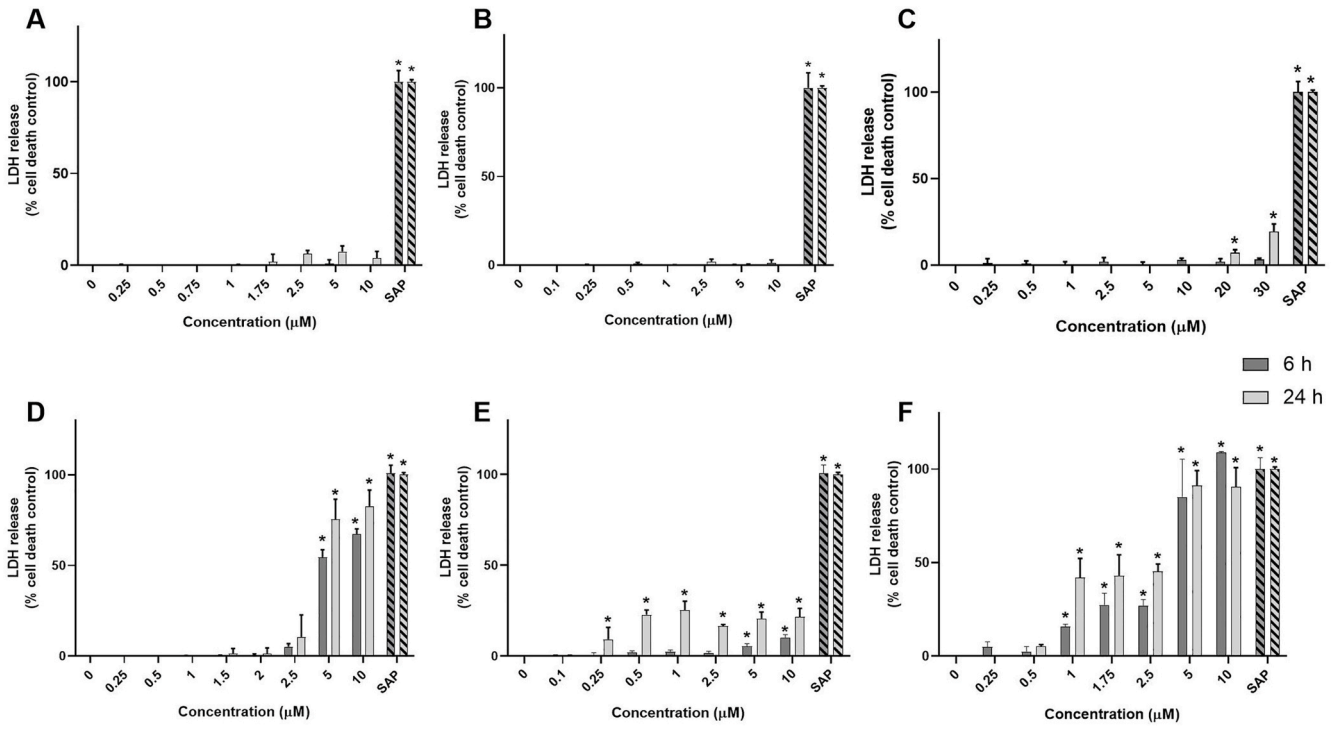


**Fig. 3. Cytotoxic effects of regulated and non-regulated mycotoxins on SH-SY5Y cells.** Neuroblastoma cells were treated with mycotoxins for 6 and 24 h and cytotoxicity was determined by LDH assay. (A) DON, (B) FB1, (C) ZEA, (D) ENN A, (E) ENN B, (F) BEA. SAP (saponin) was used as cell death control. Data are mean ± SEM of three independent replicates. Data expressed in percentage of cell death control cells. Statistical differences determined by Student's *t*-test. *p ≤ 0.05 compared to control cells.

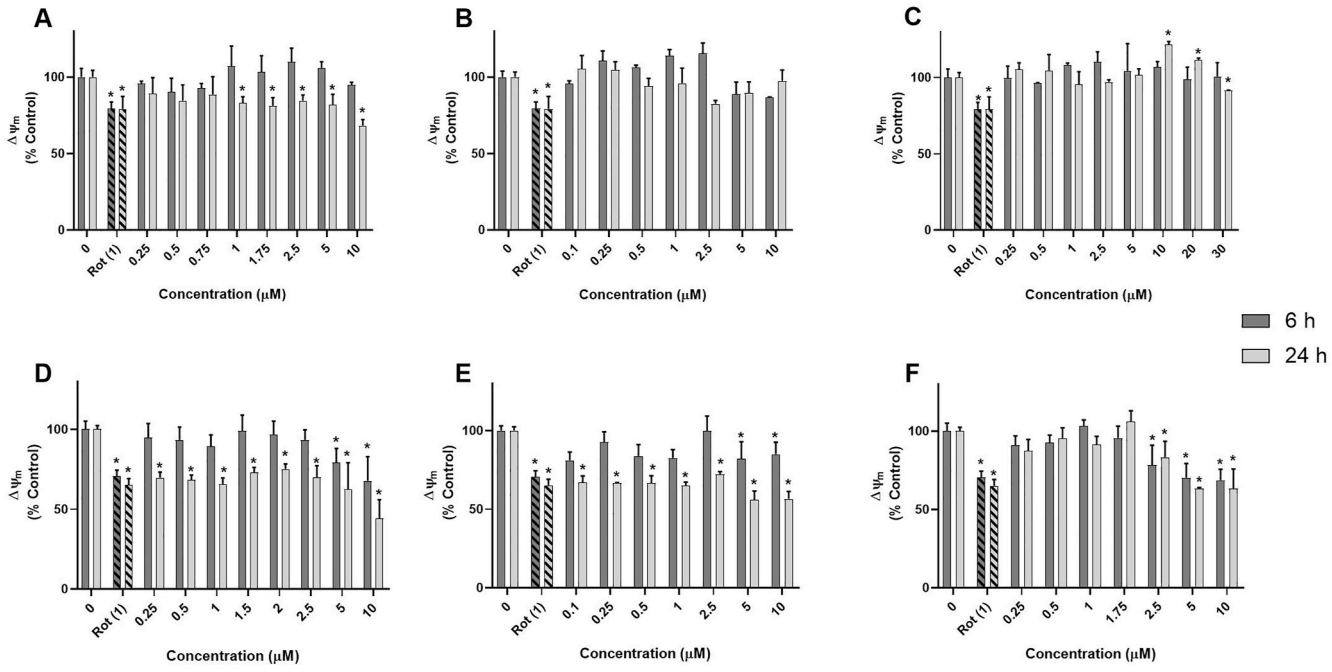


**Fig. 4. Mitochondrial membrane potential of human neuroblastoma cells after treatment with regulated and emerging mycotoxins.** (A) DON, (B) FB1, (C) ZEA, (D) ENN A, (E) ENN B, (F) BEA. Cells were treated for 6 and 24 h and $\Delta\Psi_m$ was determined by TMRM assay. Data are the mean ± SEM of three independent replicates, expressed as percentage of control cells and compared to untreated control cells by Student's *t*-test. *p ≤ 0.05.

emerging toxins produced a greater cell damage than the regulated ones, with the exception of DON.

Next, cytotoxicity of mycotoxins was determined in the human neuroblastoma cell line with LDH assay. LDH release to the culture medium provides information about the degree of cell membrane damage. SAP at 1 mg/mL was used as membrane rupture control. Cells were treated with mycotoxins at the same concentrations used in MTT assay. Due to the similar results obtained in MTT assay at 24 and 48 h of incubation, LDH experiment was performed at 6 and 24 h. No cytotoxic effects were observed after treatment with the regulated mycotoxins

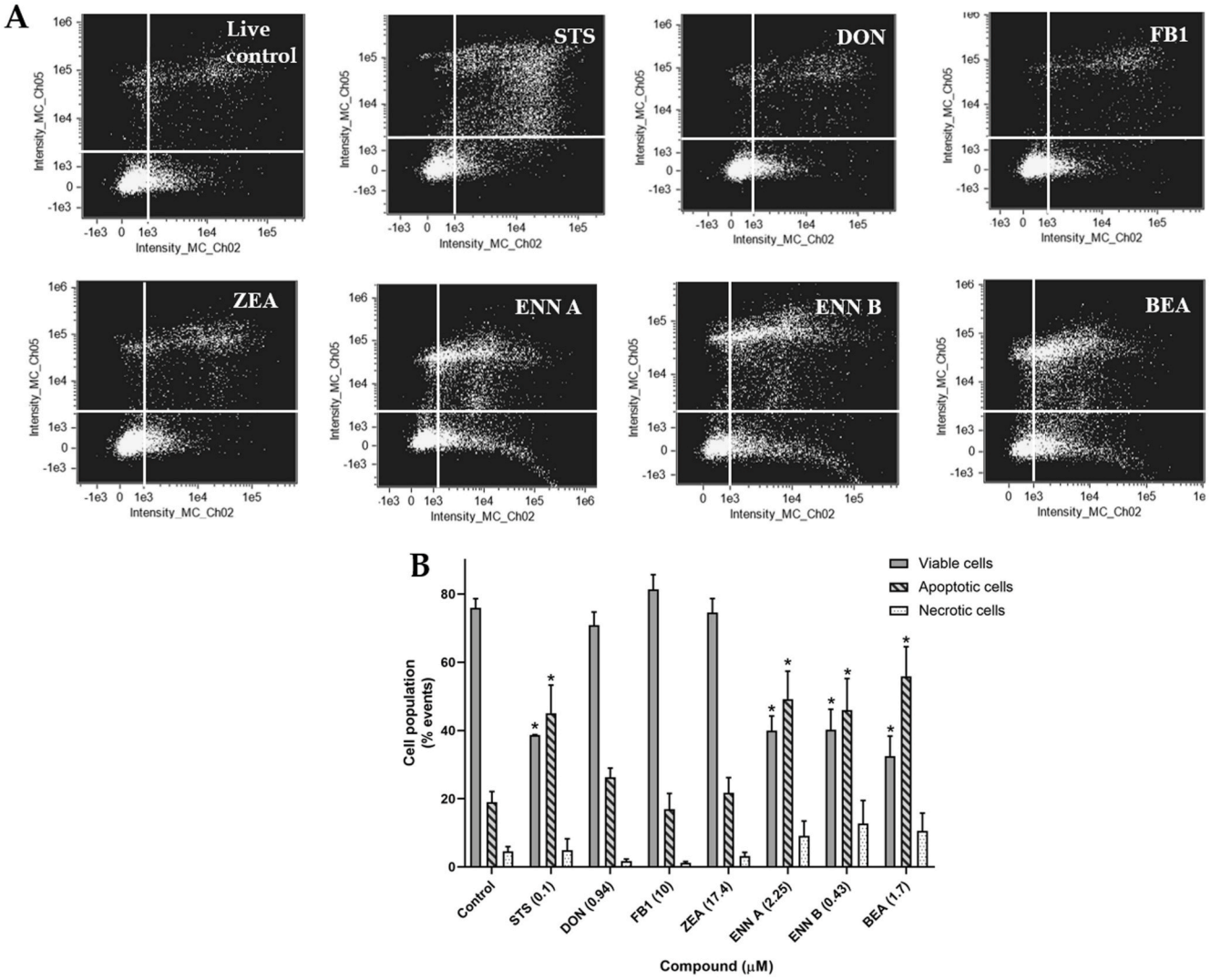


**Fig. 5. Analysis of cell death type induced by regulated and emerging mycotoxins in SH-SY5Y cell line.** Cells were treated with mycotoxins for 24 h and co-stained with Annexin V-FITC and PI. STS was used as positive control and fluorescence was analysed by flow cytometry. (A) Representative scatter plot of each treatment. X-axis represents PI fluorescence, y-axis represents Annexin V-FITC fluorescence. Data are expressed as percentage of total events analysed. Percentages of viable cells (Annexin V-FITC -/PI -), apoptotic cells including early apoptosis (Annexin V-FITC + /PI –) and late apoptosis (Annexin V-FITC + /PI +), and necrotic cells (Annexin V-FITC –/PI+) were calculated. (B) Bar graph of the type of cell death induced by mycotoxins. Data are mean ± SEM of three independent experiments, compared to control cells by Student's *t*-test. *$p \leq 0.05$.

DON and FB1 (Fig. 3A and B). In the case of ZEA, a slight increase in LDH leakage was found at 20 and 30 μM (9.1 ± 8.9% and 19.5 ± 4.3%, respectively) after 24 h (Fig. 3 C). On the other hand, ENN A significantly increased LDH release of SH-SY5Y cells at 5 and 10 μM at both incubation times, presenting $EC_{50}$ values of 3.9 μM (CI: 3.6–4.2 μM) and 3.5 μM (CI: 2.8–4.4 μM) at 6 and 24 h, respectively (Fig. 3 D, Figure S2). ENN B only showed significant effects on LDH release after 24 h of treatment, producing an increase about 20% at concentrations above 0.25 μM (Fig. 3 E). Finally, the cytotoxic effect produced by BEA was greater than the originated by the other mycotoxins used in this study, inducing LDH leakage at concentrations over 1 μM (Fig. 3 F). $EC_{50}$ was calculated after 6 and 24 h of exposure, and this mycotoxin presented values of 2.9 μM (CI: 1.9–4.6 μM) and 2.0 μM (CI: 1.4–2.9 μM), respectively (Figure S2).

In view of the differences observed in MTT and LDH assay, the study was followed by assessing the effect of the selected mycotoxins on $\Delta\Psi_m$. SH-SY5Y cells were treated at the same concentrations used in the previous assays for 6 and 24 h, and TMRM assay was performed. The assay was validated by using the inhibitor of the electronic transport chain Rot at 1 μM (Zhou et al., 2018).

The regulated mycotoxin DON did not produce any effect at 6 h, but it reduced $\Delta\Psi_m$ between 16.9 and 34.0% at concentrations higher than 1 μM after 24 h of treatment (Fig. 4 A). On the other hand, treatment with FB1 did not generate significant changes in $\Delta\Psi_m$ of SH-SY5Y cells at both incubation times. Finally, ZEA produced a slight decrease at 30 μM after 24 h of incubation (Fig. 4B and C). Regarding emerging mycotoxins, ENN A produced significant differences compared to control cells at 5 and 10 μM after 6 h of incubation (79.0 ± 8.9% and 67.7 ± 14.5%, respectively) (Fig. 4 D). At 24 h, this emerging toxin affected to $\Delta\Psi_m$ at all the concentrations tested. The same was observed with ENN B treatment, which altered $\Delta\Psi_m$ at the highest doses employed (5 and 10 μM) after 6 h of incubation and caused significant differences with respect to the control at all the concentrations assayed at 24 h (Fig. 4 E). Regarding BEA, it depolarized the mitochondrial membrane at concentrations ranging 2.5–10 μM at both incubation times (Fig. 4 F). In all the experiments, Rot produced a depolarization of the mitochondria, resulting in percentages about 75% of control cells.

Finally, the type of death caused by single mycotoxins in SH-SY5Y cells was evaluated. In view of the previous results, cells were treated with mycotoxins at $IC_{50}$ concentrations obtained with MTT test for 24 h.

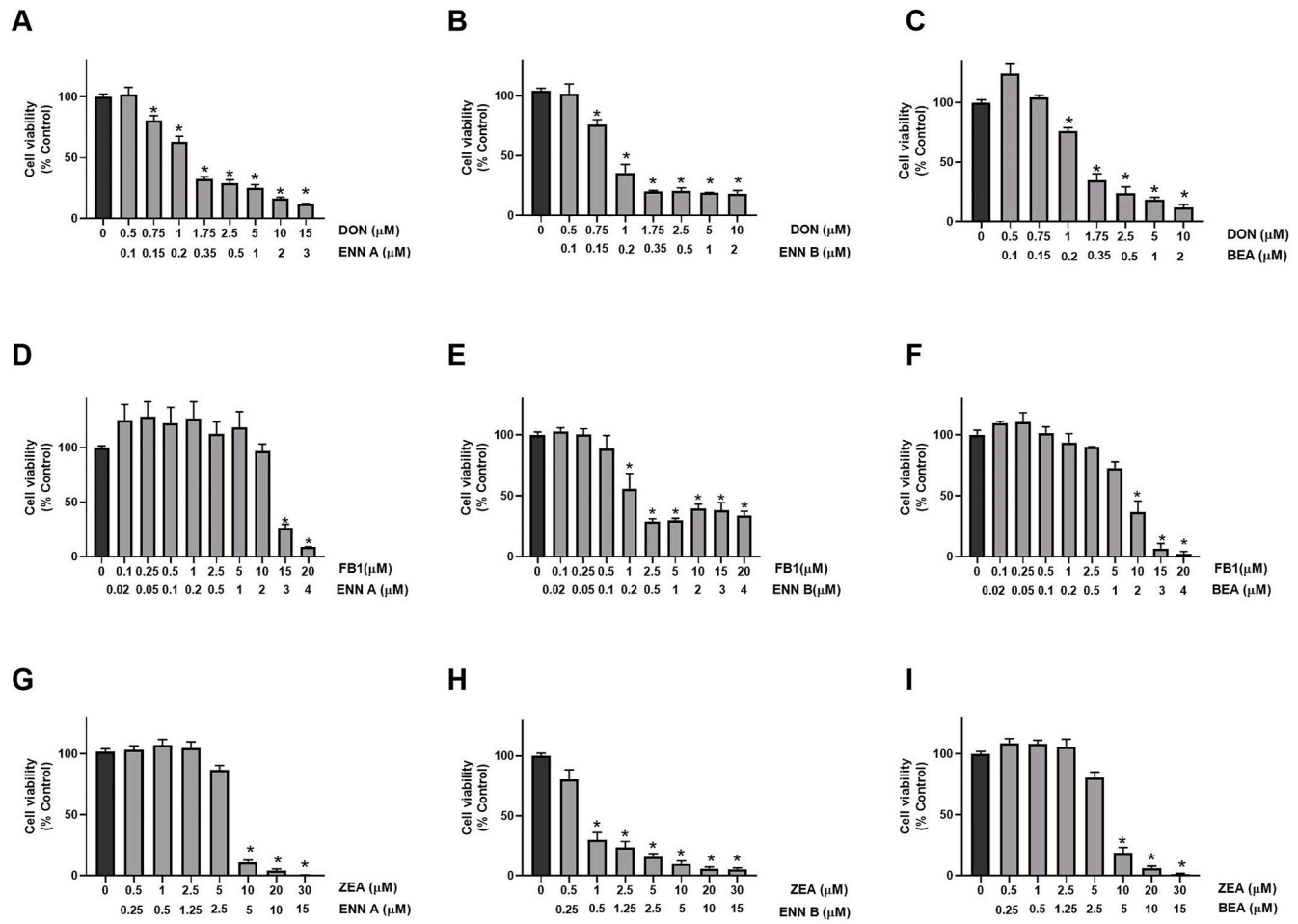


**Fig. 6. Effect of regulated and emerging mycotoxin binary combinations in cell viability.** SH-SY5Y cells were treated with binary mixtures of regulated and non-regulated mycotoxins for 24 h and MTT assay was carried out. (A) DON + ENN A (5:1), (B) DON and ENN B (5:1), (C) DON + BEA (5:1), (D) FB1+ ENN A (5:1), (E) FB1 + ENN B (5:1), (F) FB1+ BEA (5:1), (G) ZEA + ENN A (2:1), (H) ZEA + ENN B (2:1), (I) ZEA + BEA (2:1). Mean ± SEM of three independent replicates performed by triplicate. Data are expressed as percentage of control cells and compared to untreated control cells by Student's *t*-test. *$p \leq 0.05$.

In the case of FB1, cells were treated at the highest dose used in the previous assays, 10 μM. For validating this assay, the known apoptotic inductor STS at 0.1 μM was used as positive control. Then, co-staining with Annexin V- FITC and PI was performed, and the fluorescence was analysed by flow cytometry (Fig. 5). The percentages of apoptotic cells, including early apoptotic cells (Annexin V-FITC positive and PI negative) and late apoptotic cells (Annexin V-FITC positive and PI positive), as well as necrotic cells (Annexin V-FITC negative and PI positive) were calculated.

As expected, 0.1 μM STS produced an increase in apoptotic cell death, reaching a percentage of 45.1 ± 5.5%. The regulated mycotoxins DON, FB1 and ZEA did not produce a significant increase in cell death at the concentrations tested (Fig. 5A and B). On the other hand, ENN A, ENN B and BEA significantly reduced the percentage of viable cells, producing an increase in apoptotic cell death of 49.2 ± 8.2%, 46.0 ± 9.3% and 55.9 ± 8.6%, respectively (Fig. 5 B).

### *3.2. Evaluation of binary mycotoxin combinations on viability and mitochondrial function of SH-SY5Y cells*

In order to mimic the co-occurrence of mycotoxins in food, regulated and emerging mycotoxins were binary combined. Concentration ratios were selected based on the mycotoxin proportions frequently found in local contaminated samples (González-Jartín et al., 2020). DON and FB1 were combined with emerging mycotoxins (ENN A, ENN B and BEA) at 5:1 ratio, whilst ZEA was combined at 2:1 ratio. In view of the effects obtained in the previous experiments, 24 h was chosen as incubation time for mycotoxin combinations.

Firstly, the effect of binary mixtures on cell viability was determined. The results obtained with DON + ENN A combination were similar to the effect produced by DON alone, causing cell damage at concentrations higher than 0.75 μM + 0.15 μM (Fig. 6 A). Regarding DON + ENN B and DON + BEA, the results were quite similar to the combination of DON + ENN A. An acute decrease was observed after treatment with DON + ENN B at 1 μM + 0.2 μM, followed by a stabilized effect at the highest concentrations used (Fig. 6 B). In the case of DON + BEA, a dose-response effect was observed, with significant results from 1 μM + 0.2 μM (Fig. 6 C).

FB1 combinations with ENN A, ENN B and BEA showed a decrease in cell viability in all cases (Fig. 6D–F). FB1 mixture with ENN A presented significant results at concentrations above 15 μM FB1 + 3 μM ENN A (Fig. 6 D). Regarding FB1+ ENN B, this combination seemed to be more harmful, producing cell damage at concentrations over 1 μM + 0.2 μM (Fig. 6 E). Finally, FB1+BEA produced significant effects at the three highest concentrations tested (Fig. 6 F).

In the case of ZEA, its combination with ENN A reduced cell viability a percentage of 89.6 ± 2.5% at concentrations over 10 μM + 5 μM (Fig. 6 G). The combination with ENN B produced an inhibition of cell viability between 69.8 and 94.6% at concentrations over 1 μM ZEA and 0.5 μM ENN B (Fig. 6 H). At last, ZEA + BEA combination had a similar effect than ZEA + ENN A, inducing cell damage at concentrations from 10 μM + 2 μM (Fig. 6 I).

**Table 2**
**$IC_{50}$ values and combination index of binary mixtures of regulated and non-regulated mycotoxins**. $IC_{50}$ values were used for combination index determination. DON: deoxynivalenol, FB1: fumonisin B1, ZEA: zearalenone, ENN A: enniatin A, ENN B: enniatin B; BEA: beauvericin, CI: 95% confidence interval.

| Binary combination | $IC_{50}$ (μM) | | Combination index |
|---|---|---|---|
| **DON + ENN A (5:1)** | **DON** 1.6 (CI: 1.4–1.9) $R^2$: 0.86 | **ENN A** 0.32 (CI: 0.27–0.38) | 1.9 |
| **DON + ENN B (5:1)** | **DON** 0.83 (CI: 0.74–0.89) $R^2$: 0.95 | **ENN B** 0.16 (CI: 0.15–0.18) | 1.3 |
| **DON + BEA (5:1)** | **DON** 1.0 (CI: 0.76–1.2) $R^2$: 0.97 | **BEA** 0.20 (CI: 0.15–0.24) | 1.2 |
| **FB1 + ENN A (5:1)** | **FB1** NA $R^2$: 0.89 | **ENN A** 2.3 (CI: 2.0–2.6) | NA |
| **FB1 + ENN B (5:1)** | **FB1** NA $R^2$: 0.89 | **ENN B** 0.15 (CI: 0.12–0.20) | NA |
| **FB1 + BEA (5:1)** | **FB1** NA $R^2$: 0.95 | **BEA** 1.4 (CI: 1.2–1.6) | NA |
| **ZEA + ENN A (2:1)** | **ZEA** 6.6 (CI: 6.1–7.2) $R^2$: 0.99 | **ENN A** 3.3(CI: 3.0–3.6) | 1.9 |
| **ZEA + ENN B (2:1)** | **ZEA** 1.0 (CI: 0.8–1.2) $R^2$: 0.86 | **ENN B** 0.5 (CI: 0.4–0.6) | 1.2 |
| **ZEA + BEA (2:1)** | **ZEA** 6.5 (CI: 5.8–7.2) $R^2$: 0.86 | **BEA** 3.3 (CI: 2.9–3.7) | 2.1 |

With the results obtained, $IC_{50}$ values for each toxin in binary combination were determined (Table 2, Figure S3). Next, combination index was calculated following Chou & Talalay method (Chou, 2010). If combination index value is higher than 1, it indicates antagonism, if it is equal to 1, it indicates additivity, and if the value is lower than 1, it indicates synergistic effect. For DON mixture with emerging toxins, the combination index obtained was higher than 1, indicating antagonism. With regard to FB1, the combination index could not be calculated due to its lack of effect in MTT assay. In the case of ZEA, its $IC_{50}$ values were lower in combination (among 1–6.6 μM) than the obtained with ZEA alone (17.4 μM). However, emerging toxins in binary mixture with ZEA presented higher $IC_{50}$ values than the toxins alone, so combination index calculation, with values above 1, revealed an antagonistic effect.

Then, the cytotoxicity of binary mycotoxin combinations was determined with LHD assay. In the case of DON, concentrations above 5 μM of this toxin in mixture with ENN A and BEA showed significant differences with the control, with LDH levels between 8.4 and 48.2% in the case of ENN A, and levels between 16.9 and 25.5% for DON + BEA mixtures. Regarding DON + ENN B mixture, only the highest concentration used (10 μM + 2 μM) induced LDH leakage (13.4 ± 3.9%) (Fig. 7A–C). FB1 combinations had a similar effect on LDH release than the reduction observed in cell viability, showing LDH leakage in all cases. FB1+ENN A reduced cell survival at the highest concentrations, starting at 10 μM + 2 μM (Fig. 7 D). In this assay, FB1 presented an $EC_{50}$ of 12.0 μM (CI: 10.8–14.8 μM), whilst ENN A $EC_{50}$ was 2.4 μM (CI: 2.1–2.9 μM) (Figure S4 A). FB1+ ENN B induced a slight and sustained cytotoxicity at concentrations higher than 1 μM + 0.2 μM (Fig. 7 E), whilst FB1+ BEA produced a dose-dependent effect at concentrations higher than 5 μM + 1 μM (Fig. 7 F). For the latter mixture, FB1 presented an $EC_{50}$ of 8.1 μM (CI: 5.3–12.2 μM) and BEA showed a value of 1.6 μM (CI: 1.1–2.4 μM) (Figure S4 B). ZEA combinations also induced an increase on LDH release comparable to the results obtained in MTT assay, without producing total membrane rupture in any case (Fig. 7G–I).

Then, TMRM assay was carried out with mycotoxin combinations to determine their effect on $\Delta\Psi_m$ (Fig. 8). DON + ENN A mixture significantly affected $\Delta\Psi_m$ at the highest concentration tested (15 μM + 3 μM), producing a decrease of 30.3 ± 5.9% (Fig. 8 A). Otherwise, DON + ENN B altered $\Delta\Psi_m$ from 0.75 μM + 0.15 μM to the highest concentrations used (Fig. 8 B). In the case of DON + BEA mixture, the mitochondrial membrane was depolarized at all the concentrations tested, with percentages between 69.8 and 81.0% with respect to control cells (Fig. 8 C). Regarding FB1 combinations, only the mixture with ENN A and ENN B had effects on $\Delta\Psi_m$ at the highest doses used (Fig. 8 D, E). Finally, ZEA + ENN A decreased $\Delta\Psi_m$ at concentrations over 5 μM ZEA and 2.5 μM ENN A (Fig. 8 G). When ZEA was mixed with ENN B and BEA, $\Delta\Psi_m$ was affected at the highest concentrations assayed, reaching levels of 60.6 ± 8.8% and 64.7 ± 9.8% at 30 μM + 15 μM (Fig. 8 H, I). As previously described in individual mycotoxin treatments, 1 μM Rot was used to validate the assay. At this concentration, Rot depolarized mitochondrial membrane, with percentages between 72.0 and 84.7% with respect to control cells.

### *3.3. Analysis of mycotoxin content and cytotoxicity of a contaminated cow feed sample and two milk extracts*

Since mycotoxin co-occurrence is frequent in animal-derived products, two different matrixes were tested in order to analyse the toxicity of real concentrations. A cow feed contaminated extract, as well as two contaminated commercial milk extracts were analysed in SH-SY5Y cells. Firstly, mycotoxin content was determined. In brief, the samples were extracted by using a QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction procedure and analysed by ultra-high liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). In the cow feed extract, it was found the presence of several mycotoxins (Fig. 9), which were quantified using linear 1/X calibration curves, results were corrected for the recovery and the matrix effect. In this sense, the sample contained 118.3 μg/kg of DON, 145.7 μg/kg of FB1, 103.2 μg/kg of FB2, 72.8 μg/kg of ZEA, 17.0 μg/kg of ENN A, 25.0 μg/kg of ENN A1, 134.1 μg/kg of ENN B, 74.1 μg/kg of ENN B1 and 76.3 μg/kg of BEA.

On the other hand, several mycotoxins were also found in milk samples (Fig. 10). They were quantified using linear 1/X calibration curves too. The milk extract **1** contained 0.37 μg/kg of ENN A, 0.42 μg/kg of ENN A1, 0.15 μg/kg of ENN B and 0.94 μg/kg of BEA, while the milk extract **2** contained 0.36 μg/kg of ENN A, 0.40 μg/kg of ENN A1, 0.14 μg/kg of ENN B, 0.30 μg/kg of ENN B1 and 0.52 μg/kg of BEA.

Then, cow feed and milk extracts were tested in human neuroblastoma cells. The cow feed extract was concentrated 20 times and its effects on cell viability and cytotoxicity were determined with MTT and LDH, respectively. Cells were treated with the concentrated extract at dilutions between 1:500 and 1:10000 for 24 h. In MTT assay, the highest concentrations used (1:500 and 1:1000) induced a significant cell damage, with levels of 39.1 ± 3.9% and 81.1 ± 1.9% with respect to control cells (Fig. 11 A). LDH release was increased in more than a 50% at the highest concentration used (Fig. 11 B), which agrees with the results obtained in MTT test.

Then, to validate if the results obtained with the extract could be compared to commercial mycotoxin standard mixture, the highest concentration of the extract (1:500) was chosen to calculate mycotoxin concentrations. SH-SY5Y human neuroblastoma cells were treated with the combination of 4.0 nM DON, 2.0 nM FB1, 2.3 nM ZEA, 0.25 nM ENN A, 2.0 nM ENN B and 0.97 nM BEA for 24 h. Also, single mycotoxin treatments at the calculated concentrations were added as control. No significant differences were observed in both MTT and LDH assays (Fig. 11C and D). Mycotoxins were also tested at higher concentrations, in order to assess their combined effect at doses included in the range of our previous assays. Therefore, the concentrations found in the extract

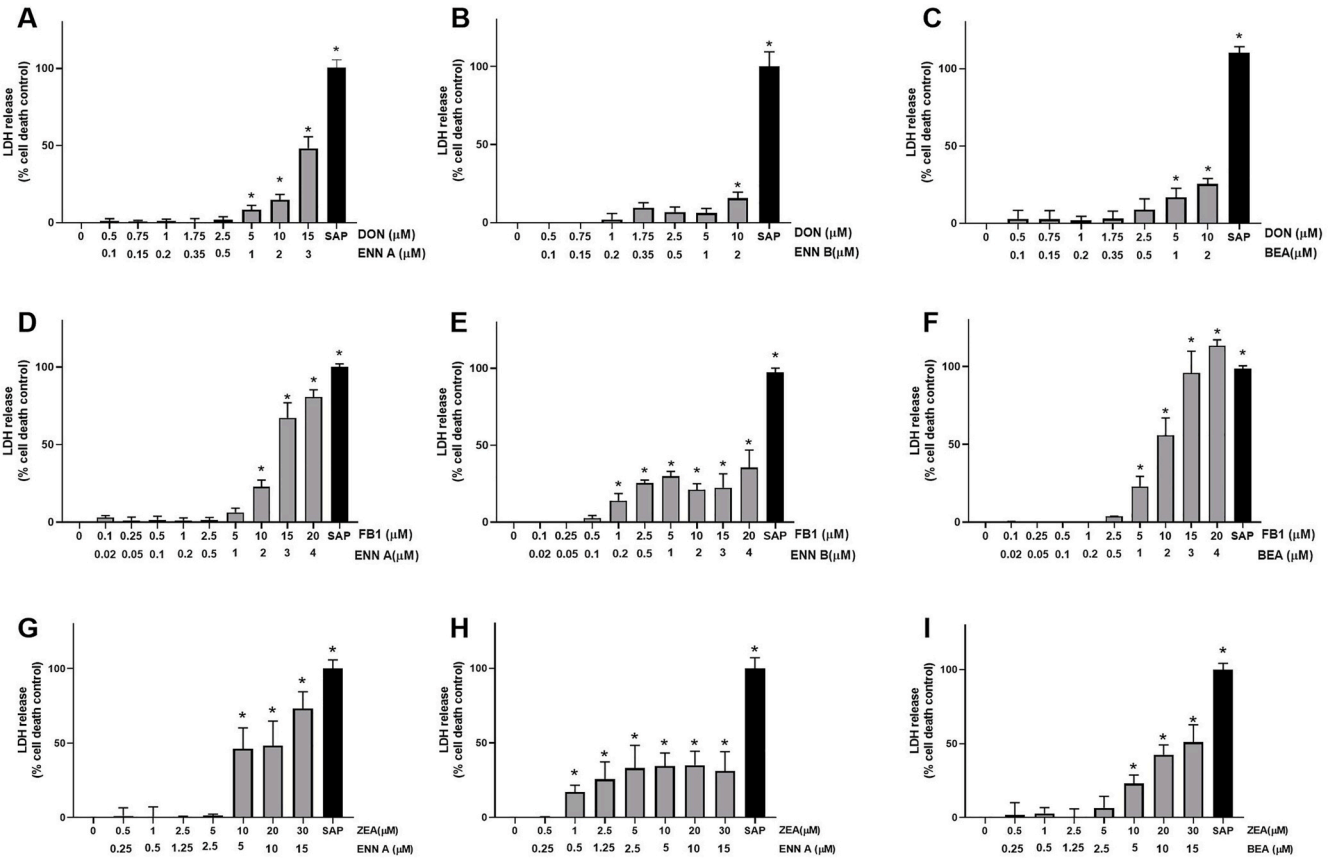


**Fig. 7. Effect of regulated and emerging mycotoxin combinations in SH-SY5Y cells.** Cells were treated with binary mycotoxin combinations. (A) DON + ENN A (5:1), (B) DON + ENN B (5:1), (C) DON + BEA (5:1), (D) FB1 + ENN A (5:1), (E) FB1 + ENN B (5:1), (F) FB1 + BEA (5:1), (G) ZEA + ENN A (2:1), (H) ZEA + ENN B (2:1), (I) ZEA + BEA (2:1). SAP (saponin) was used as cell death control. Data are mean ± SEM of three independent experiments, expressed as percentage of cell death control cells. Statistical differences determined by Student's *t*-test. *$p \leq 0.05$.

were increased 1000 times, keeping the mycotoxin ratio present in the contaminated sample (Fig. 11E and F). In this case, 4.0 μM DON and 2.0 μM ENN B presented the most remarkable effects, decreasing cell viability by 81.9 ± 0.89% and 67.8 ± 6.0%, respectively. BEA at 0.97 μM also produced a slight decrease on the cell activity (11.9 ± 4.9%). Finally, when the mixture of all mycotoxins was added, cell viability was reduced to a percentage of 11.1 ± 1.5% of control cells (Fig. 11 E). However, when LDH assay was performed, the mix of all mycotoxins only increased LDH release by 13.7 ± 0.5%, a rise that seems to be mediated by the effect of DON, ENN B and BEA, which elevated LDH leakage by 11.3 ± 3.7%, 32.5 ± 2.6% and 22.2 ± 10.0%, respectively (Fig. 11 F).

Finally, the milk natural contaminated extracts were tested, in the same way as was done with the cow feed sample. Milk samples were chosen because they did not content regulated mycotoxins and had lower concentrations of ENNs and BEA than those present in the cow feed sample. The extracts were concentrated 20 times and its effects on cell viability and cytotoxicity were determined with MTT and LDH, respectively (Fig. 12A and B). Cells were treated with the concentrated extract at dilutions between 1:500 and 1:10000 for 24 h. With milk extract **1**, no differences were observed with respect to control cells neither on MTT nor LDH assays. However, with milk extract **2**, a reduction on cell viability of 14.0 ± 6.8% was observed at the highest concentration used (1:500), although no significant differences in LDH release were detected. It should be notes that the main difference between these two samples is the presence of ENN B1 in the extract **2**.

## 4. Discussion

Mycotoxin contamination is a global problem, the Food and Agriculture Organization of the United Nations estimates that approximately 25% of the cereals produced in the world are contaminated by mycotoxins (Janik et al., 2020). Contaminated food consumption supposes an important risk to human health, as mycotoxins are known to produce illnesses such as cancer, kidney pathologies and neurological diseases (Marin et al., 2013). In this sense, several *in vitro* and *in vivo* studies have demonstrated that mycotoxins can cross the BBB due to their physical properties, targeting the brain (Bonnet et al., 2012; Osuchowski et al., 2005b; Taevernier et al., 2016). In this study, a human neuroblastoma cell line, SH-SY5Y cells, was chosen in an attempt to understand how mycotoxin neurotoxicity occurs, with an especial focus on emerging mycotoxins because data about their neurotoxic effect are scarce. In fact, there are not established maximum legal limits for these toxins in food and feed due to the lack of information about their toxicity (EFSA, 2014).

Firstly, the individual effects of regulated and emerging toxins on cell survival and mitochondrial function were analysed. Regarding regulated mycotoxins, DON produced the greatest cell damage, with an $IC_{50}$ of 0.94 μM. ZEA presented an $IC_{50}$ value almost 20-fold greater (17.4 μM), whilst FB1 did not display toxic effects in this cell line up to 10 μM. However, when membrane leakage and cell death type were analysed, DON and ZEA did not show harmful effects on human neuroblastoma cells. Considering that MTT is metabolised by mitochondria and is a more sensitive assay than LDH (Fotakis and Timbrell, 2006), these results could indicate that mitochondria is being affected by DON and ZEA, but plasmatic membrane integrity is not compromised at the

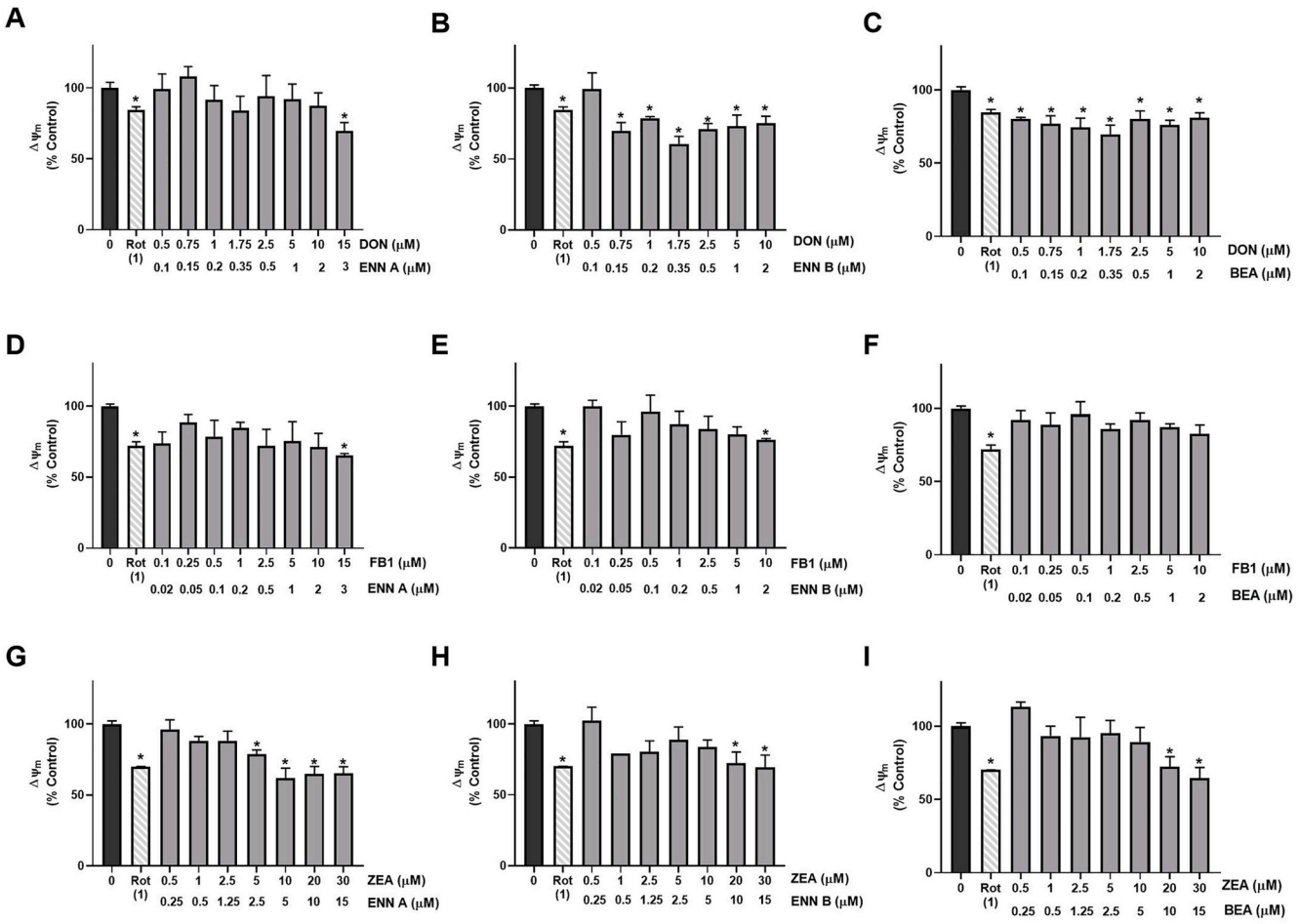


**Fig. 8. Effect of regulated and emerging mycotoxin binary combinations in $\Delta\Psi_m$ of SH-SY5Y cells.** Cells were treated with mycotoxin mixtures for 24 h, and TMRM assay was performed to evaluate their effect on $\Delta\Psi_m$ (A) DON + ENN A (5:1), (B) DON + ENN B (5:1), (C) DON + BEA (5:1), (D) FB1 + ENN A (5:1), (E) FB1 + ENN B (5:1), (F) FB1 + BEA (5:1), (G) ZEA + ENN A (2:1), (H) ZEA + ENN B (2:1), (I) ZEA + BEA (2:1). Data are mean ± SEM of three independent replicates, expressed as percentage of untreated control cells. Statistical differences determined by Student's *t*-test. *p ≤ 0.05.

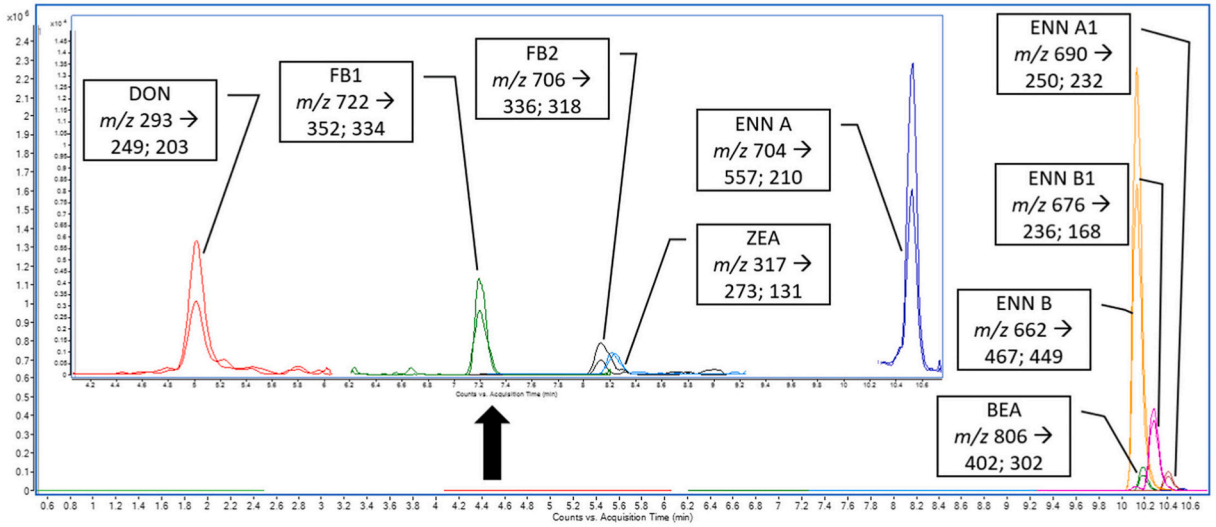


**Fig. 9. UHPLC-MS/MS chromatogram of the cow feed extract.** Compounds were identified by using the *m/z* of the parent compound and two product ions.

concentrations tested.

DON neurotoxicity has been previously reported both *in vitro* and *in vivo* (Zhang et al., 2020) and recent data confirms that this toxin targets mitochondria and generates oxidative stress (Huang et al., 2019). Furthermore, at a concentration of 100 μM, DON has been shown to induce mitochondria-dependent apoptotic death through mitochondrial

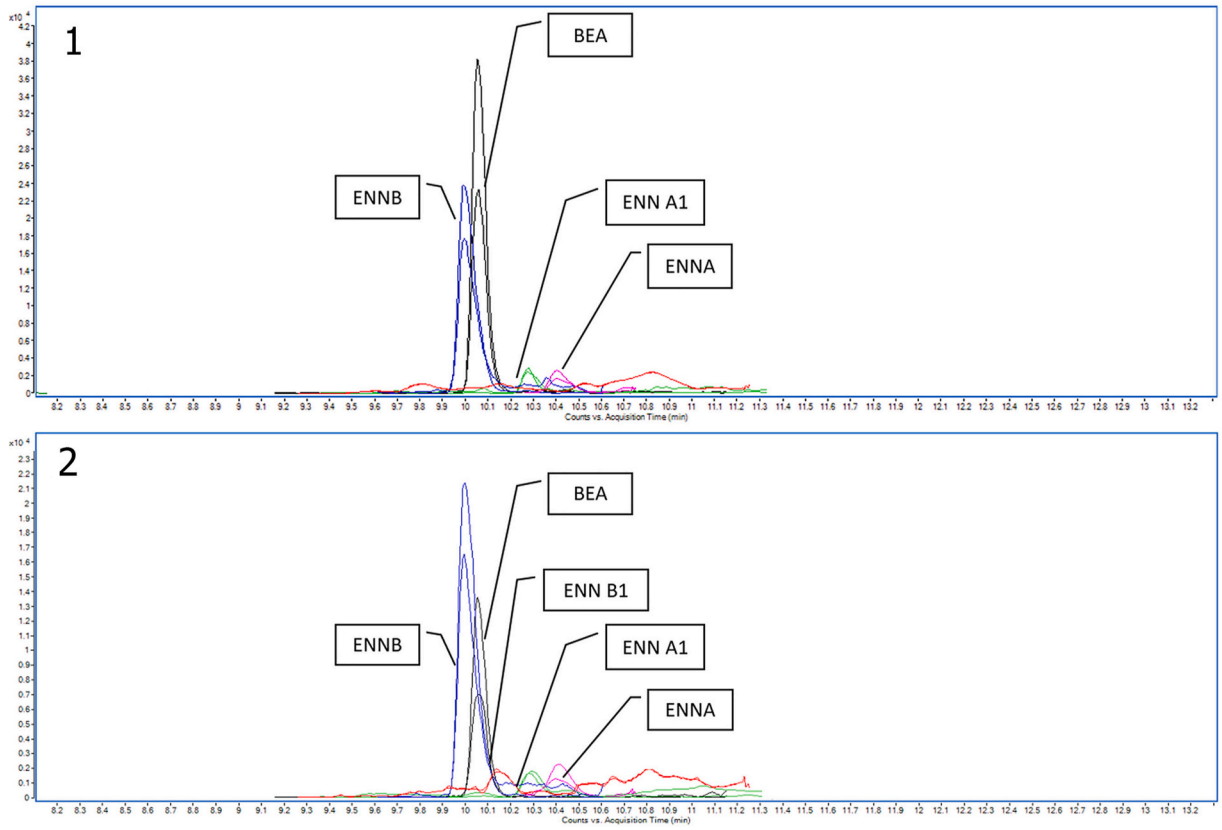


**Fig. 10. UHPLC-MS/MS chromatogram of the milk extracts.** Compounds were identified by using the *m/z* of the parent compound and two product ions.

permeability transition pore opening in human carcinoma cells (Bensassi et al., 2012). However, apoptosis cannot be confirmed in our report, because a lower dose (0.94 μM) and a different cell type were used in flow cytometry assay. Nevertheless, a decrease in $\Delta\Psi_m$ was observed at concentrations between 1 and 10 μM after 24 h of incubation, indicating that mitochondria were being affected by this toxin. This effect could be related to the mitochondrial permeability transition pore, whose opening can be reversible. Only if this opening is massive and sustained, it leads to apoptotic cell death (Panel et al., 2018), so the effect observed with DON in TMRM assay could be related to a transient mitochondrial permeability transition pore opening, which is not leading to apoptosis.

In the case of FB1, our results indicate that this toxin does not affect to cell viability and mitochondrial function of SH-SY5Y cells, agreeing with previous works (Osuchowski and Sharma, 2005). FB1 is considered neurotoxic due to their involvement in equine leukoencephalomalacia because it induces demyelination and sphinganine elevation. However, there is no evidence of FB1 cytotoxicity in neuronal lines, which could be related to the absence of myelin in cultured cells (Osuchowski and Sharma, 2005). Moreover, it has been hypothesized that its neurotoxic effect could be due to its ability to activate glial cells (Osuchowski et al., 2005a). Regarding ZEA, our results are similar to the data presented by Venkataramana et al. (2014) in SH-SY5Y cells, which reported that this toxin diminished cell viability at concentrations lower than 25 μM. Moreover, the results presented in our work confirm that ZEA cytotoxicity is mediated by mitochondrial dysfunction, since mitochondrial membrane is depolarized at the highest concentrations.

Emerging mycotoxins caused a significant alteration in mitochondrial function, depolarizing the mitochondrial membrane, and being cytotoxic to SH-SY5Y cells through the induction of apoptotic cell death. ENN A, ENN B and BEA presented $IC_{50}$ values of 2.2, 0.43 and 1.7 μM in cell viability, respectively. It is noteworthy the effect produced by ENN B at 6 h, because the mycotoxin induced a sudden decrease in cell viability at 0.5 μM that was sustained until 10 μM, without observing a dose-dependent effect. This response has been previously reported in Caco-2 cells and murine macrophages treated with ENN B (Gammelsrud et al., 2012; Ivanova et al., 2012). In fact, although ENN B presented the lowest $IC_{50}$ in MTT assay, when membrane leakage was assessed by LDH assay, only ENN A and BEA showed a dose-dependent response. ENN B produced again an almost constant response, with an increase on LDH release about 30%. This sustained effect could be related to the binding of ENN B to a specific receptor, which would show a high sensitivity and specificity for the toxin at low concentrations. There are not studies about potential receptors of ENN B in mammalian cells, although it is known that the toxin inhibits the yeast multidrug resistance pump Pdr5p (Hashizume and Nishimura, 2008).

Non-regulated mycotoxins also produced mitochondrial depolarization, which could be related to their ionophoric properties. ENNs and BEA are cyclic peptides which affect biological membranes by forming complexes with metals like $K^+$, $Na^+$ and $Ca^{+2}$, allowing ion transport through the membranes. Thus, mitochondrial membrane is an important target of these emerging mycotoxins (Bertero et al., 2020; Tonshin et al., 2010). In this sense, neuronal cells are very susceptible to mitochondrial malfunction due to their great energy consumption, so a slight decrease on mitochondrial potential could have a great impact on neuronal function (Yin et al., 2014). It should be noted the total inhibition of cell viability produced by ENN A and BEA at the highest doses tested, which could be associated to their effects over mitochondria, as was confirmed when $\Delta\Psi_m$ was analysed. In this assay, the effect produced by ENNs and BEA differs. ENNs affected to $\Delta\Psi_m$ at all the concentrations tested, while BEA depolarized the mitochondria in a dose-dependent manner. These dissimilarities could be due to their differences in ionophoric properties because ENNs mainly modify $K^+$ channels, while BEA mostly affects to $Ca^{+2}$channels (Tonshin et al., 2010). Finally, when the type of cell death was analysed, it was found that ENN A, ENN B and BEA were producing apoptotic cell death in SH-SY5Y human neuroblastoma cells. These

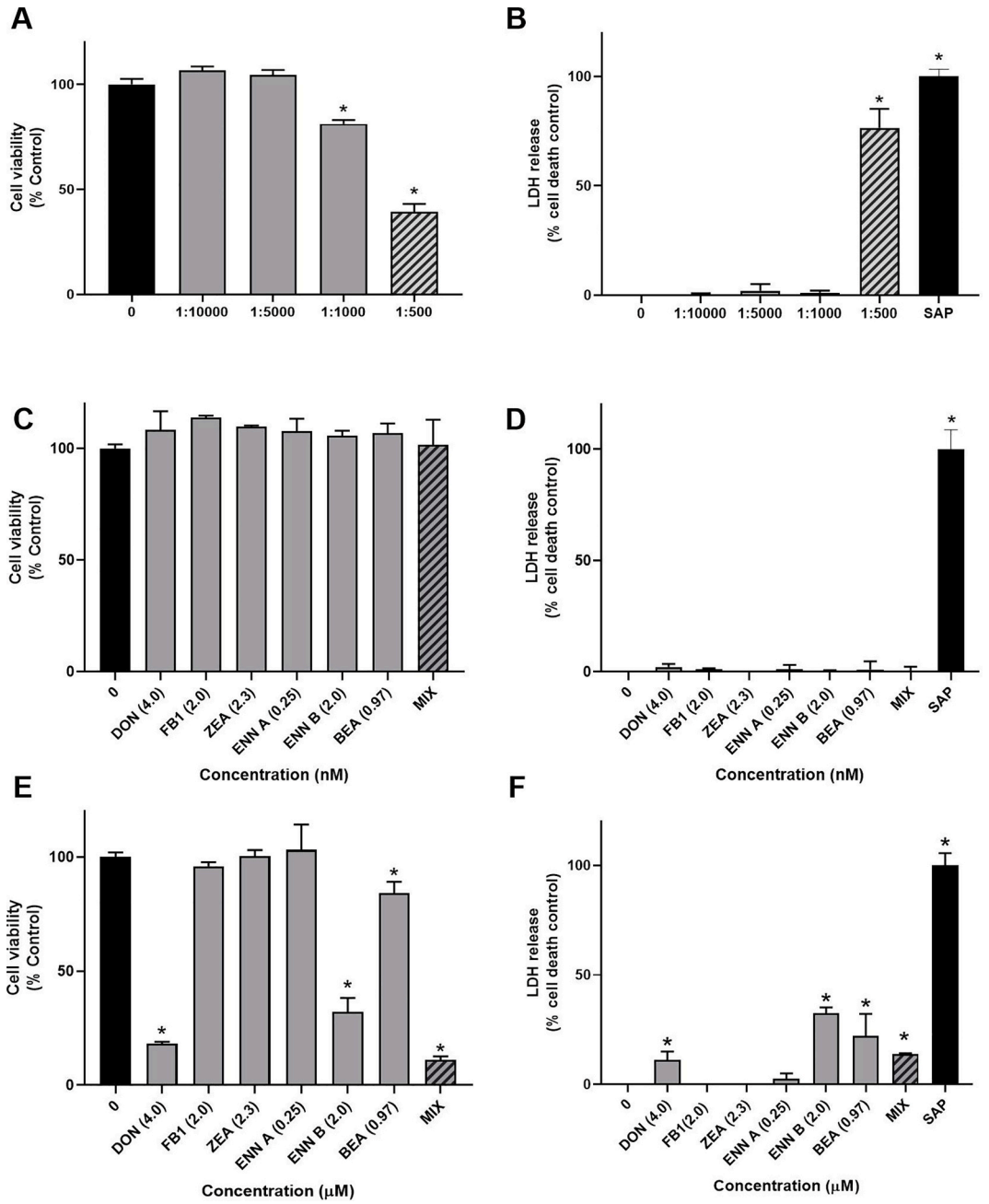


**Fig. 11.** Analysis of a cow feed contaminated extract and its equivalent mycotoxin standard mixture on cell viability and cytotoxicity of SH-SY5Y cells. Human neuroblastoma cells were treated for 24 h and MTT and LDH tests were carried out. (A) Effect on cell viability of a concentrated cow feed extract at dilutions between 1:500–1:10000. (B) Cytotoxicity, assessed by LDH assay, of SH-SY5Y cells treated with the concentrated extract. (C) Cell viability of cells treated with commercial mycotoxins, alone and in mixture, at the concentrations found in the 1:500 dilution of cow feed extract. (D) Effects of the standard mycotoxin mixture on cytotoxicity of neuroblastoma cells. (E) Cell viability of cells treated with commercial mycotoxins at concentrations 1000 times higher than those found in the 1:500 extract. (F) Cytotoxicity of SH-SY5Y cells treated with mycotoxins at doses 1000 times higher than those found in the cow feed extract. SAP (saponin) was used as cell death control. Mean ± SEM of three independent experiments. Cell viability aata are expressed as percentage of untreated control cells, whilst LDH test results are presented as percentage of cell death control cells- Statistical differences were determined by Student's *t*-test. *$p \leq 0.05$.

results agree with previous studies, which have reported these emerging mycotoxins to produce apoptosis through the mitochondrial pathway in other cell lines (Manyes et al., 2018; Prosperini et al., 2013). To our knowledge, this is the first report of ENN A and ENN B pro-apoptotic effects in neuronal cells.

With regard to BEA, its cytotoxicity has been recently reported in SH-SY5Y cells (Agahi et al., 2020a, 2020b; Juan et al., 2020; Montesano et al., 2020). These authors reported $IC_{50}$ values among 2.5–3.2 μM, in the range of the data obtained in our study. The same authors described an increase in the expression of the apoptotic gene *Bcl-2* after treatment with BEA, which agrees with our results (Agahi et al., 2020b).

In summary, the analysis of individual mycotoxins revealed that ENN B was the most damaging molecule, followed by DON, BEA, ENN A, ZEA and FB1, which did not affect to SH-SY5Y cells. With the exception of DON, our results suggest that emerging mycotoxins produce a greater mitochondrial impairment and neurotoxicity than the regulated ones and could suppose a health hazard. In this sense, EFSA experts stress that chronic exposure to emerging mycotoxins could pose a public health problem, in fact, repeated oral exposure to ENN B and BEA produced genotoxic effects in mice (Maranghi et al., 2018).

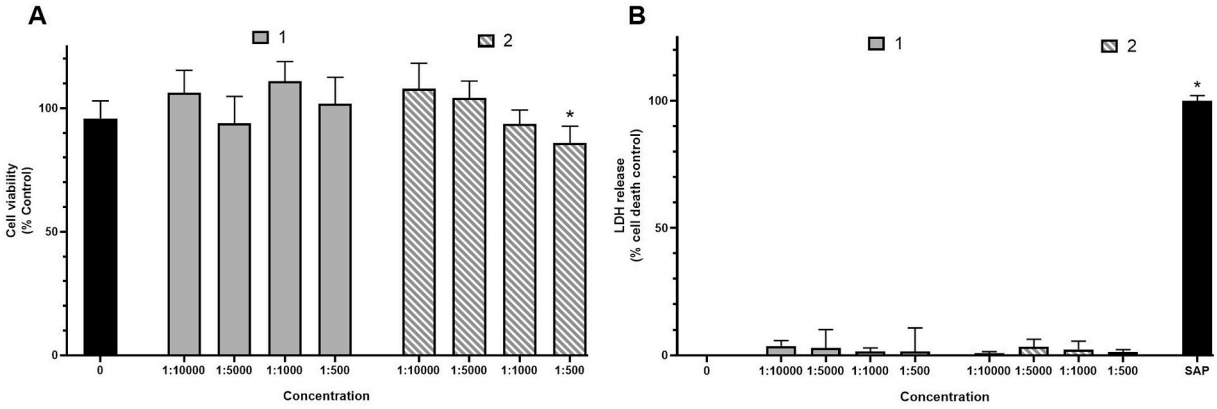


**Fig. 12. Analysis of milk contaminated extracts effect on cell viability and cytotoxicity of SH-SY5Y cells.** Human neuroblastoma cells were treated for 24 h and MTT and LDH tests were carried out. (A) Effect on cell viability of concentrated milk extracts at dilutions between 1:500–1:10000. (B) Cytotoxicity, assessed by LDH assay, of SH-SY5Y cells treated with the concentrated extracts. SAP (saponin) was used as cell death control. Mean ± SEM of three independent experiments. Data expressed as percentage of untreated control cells. Cell viability aata are expressed as percentage of untreated control cells, whilst LDH test results are presented as percentage of cell death control cells- Statistical differences were determined by Student's *t*-test. *p ≤ 0.05.

European multi-mycotoxin contamination studies indicate that 75–100% of animal feed samples contain more than one mycotoxin (Streit et al., 2012). Moreover, human bio-monitoring studies have found that 100% of human urine samples contained biomarkers of 2 or more mycotoxins, and up to 5 mycotoxins were detected in a severe case of co-exposure (Alassane-Kpembi et al., 2017). In this context, the effects of binary combinations of regulated and non-regulated mycotoxins were analysed. Antagonistic effects were found for the binary mixtures between DON, ZEA and emerging mycotoxins. Regarding FB1, although the combination index could not be calculated, the effects its mixture with emerging mycotoxins are almost the same observed with non-regulated toxins alone, suggesting an additive effect. Our results coincide with previous data in other cell types, in which antagonism was found. This is the case of DON and ENN B combination in intestinal cells (Fernández-Blanco et al., 2016; Vejdovszky et al., 2016), or BEA and DON mixture in CHO–K1 cells (EFSA, 2014).

Finally, contaminated cow feed and milk extracts were tested in our *in vitro* model. All the extracts tested were analysed by methods previously in-house validated for the quantification of regulated, emerging and modified mycotoxins. The sensitivity of the methods is high, allowing the detection of compounds at concentrations lower than the maximum residue levels established in European regulations. In addition, the accuracy and precision are good enough to fulfil the performance characteristics established in the legislation (Commission Regulation (EC) No 401/2006). In the cow feed extract, it was detected the presence of regulated and emerging mycotoxins, although in concentrations lower than the maximum levels recommended in the UE for animal feed (Commission Recommendation 2006/576/EC). It can be highlighted the presence of the emerging BEA and ENNs, which were found in low amounts. The extract decreased cell viability and generated cytotoxicity in SH-SY5Y cells, despite the fact that the regulated mycotoxins present are below the established maximum limits. When the commercial standard equivalent mixture was tested, no effects were found in cell viability. Only when commercial standards were mixed at concentrations 1000-fold higher than those found on the extract, similar results on cell viability were obtained. This may be due to the presence of other mycotoxins in the sample which could be enhancing the effect of the mycotoxins selected for this study. As ENN A1, ENN B1 and FB2 were also detected in the contaminated extract, the difference could be linked to the presence of these mycotoxins. Since the toxicity produced by the cow feed sample could be due to the presence of ENN A1 and ENN B1, two milk samples naturally contaminated with ENNs and BEA, in much lower concentrations than those found in cow feed, were tested. Both milk samples contained similar amounts of mycotoxins, being the main difference between them the presence of ENN B1 in extract **2**. This sample reduced SH-SY5Y cells viability at the highest dilution tested, whilst extract **1** did not show any significant effect on this cell line. These results indicate that ENN B1 possibly exhibits neuronal cytotoxicity and could be potentiating the effect of other mycotoxins on this cell line. In fact, ENNs combination is known to produce a greater damage than the toxins separately (EFSA, 2014), and ENN A1 and ENN B1 have displayed higher toxicity than ENN A and ENN B in several cell types (Ivanova et al., 2006; Khoshal et al., 2019; Krug et al., 2018; Prosperini et al., 2013). On the other hand, FB2 has synergistic effects with FB1 in human cells (Yu et al., 2020). Moreover, the decrease on cell viability at such low toxin concentrations suggests that matrix is influencing mycotoxin cytotoxicity. Particularly, the milk extract presented a greater reduction on cell viability than expected, indicating that the lipophilic properties of emerging mycotoxins could be related to this damaging effect. In view of these results and due to their prevalence on natural samples, it would be interesting to study the individual and combined effects of ENN A1 and B1 in this cell line in future studies. Moreover, the analysis of different contaminated matrixes such as cheese or beef would help to better understand if the presence of emerging mycotoxins in food could be considered a public health hazard, as is being suggested by our results.

In conclusion, our results denote that emerging mycotoxins and DON produce mitochondrial-mediated cytotoxicity in human neuroblastoma cells, indicating that non-regulated mycotoxins could pose a health hazard. Binary combinations of regulated and emerging mycotoxins resulted in antagonistic effects, but when they co-occur on natural matrixes, a greater damaging effect than expected is observed. In view of these results, it would be of particular interest to further study the neurotoxic mechanism of action of emerging mycotoxins and to compare their effects on different natural matrixes, which will help to better understand their impact on human health and, consequently, to decide if maximum legal limits in food should be established.

## Disclosure statement

The authors have no conflicts of interest to declare.

## CRediT authorship contribution statement

**Nadia Pérez-Fuentes:** Investigation, Writing – original draft, Writing – review & editing. **Rebeca Alvariño:** Conceptualization, Methodology, Writing – review & editing. **Amparo Alfonso:** Conceptualization, Methodology, Writing – review & editing. **Jesús González-**

**Jartín:** Investigation. **Sandra Gegunde:** Investigation. **Mercedes R. Vieytes:** Methodology. **Luis M. Botana:** Funding acquisition, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112308.

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