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RIGORÓZNÍ PRÁCE

Cytotoxicity of mycotoxins *in vitro* methods using CHO-K1 cells

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I declare that I individualy worked up this rigorous work and that I used only the mentioned literature.

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I. SOUHRN/SUMMARY

Účelem této práce bylo určit, zda mykotoxiny produkované plísní rodu *Fusarium*, mohou působit cytotoxicky na savčí buňky CHO-K1. V této studii byly testovány tři mykotoxiny produkované rodem *Fusarium*: beauvericin (BEA), deoxynivalenol (DON) a T-2 toxin. Jejich schopnost působit cytotoxicky byla hodnocena zkouškou s neutrální červení. Buňky CHO-K1 byly vystaveny různým koncentracím mykotoxinů BEA, DON a T-2 v různých inkubačních časech. Rozsah poškození buňky byl hodnocen testem s neurální červení po 24, 48 a 72 hodinové inkubaci s danými mykotoxiny.

Současně byla sledována životnost buněk, které byly vystaveny směsím dvou nebo tří jmenovaných mykotoxinů. V této práci byly zaznamenány významné rozdíly mezi testovanými směsmi.

Bylo námi prokázáno, že všechny jednotlivě testované mykotoxiny (BEA, DON, T-2 toxin) redukují životnost buněk CHO-K1. Jednotlivé mykotoxiny snižují životashopnost ve zvyšujícím se pořadí: BEA<DON<T-2 toxin. Naše výsledky ukazují, že buňky CHO-K1 jsou velmi citlivé k T-2 toxinu. T-2 vykazuje nejsilnější cytotoxickou odpověd vůči testované buněčné linii.

Získané výsledky signalizují, že buněčná linie CHO-K1, vykazuje závislot cytotoxicity na expozičním čase a koncentraci mykotoxinu. Naše výsledky demonstrují, že T-2 toxin byl shledán více cytotoxickým v průběhu času, což je v naprostém souladu s již dříve publikovanými informacemi.

Cílem této práce bylo rovněž zhodnotit cytotoxicitu nízkých koncetrací směsí mykotoxinů na buňky CHO-K1. Zabývali jsme se cytotoxickými interakcemi dvojných a trojných směsí mykotoxinů rodu *Fusarium* v působení na buňky CHO-K1. Životnost buněk byla opět měřena testem s neutrální červení. Všechny mykotoxiny (BEA, DON, T-2 toxin) testované v kominacích vykazovaly zvýšený pokles v životnosti buněk ve srovnání s hodnotami získanými individuálním testováním.

Nárůst cytotoxicity byl pozorován pro BEA pro 24 až 72 hodinovou expozici, když byl kombinovám s T-2 toxinem, ve srovnání se s hodnotami získanými individuálně testovaným BEA.

Kombinace BEA s DON vykazuje lehké zvýšení cytotoxicity během 24 až 72 hodinové expozice v porovnání s výsledky jednotlivě testovaného DON. Můžeme shrnout, že DON neměl žádný přídavný účinek na redukci životnosti buněk, když byl testován v kombinaci s BEA po dobu 24 až 72 hodin, v porovnání s jednotlivě testovaným DON. Pokles životnosti buněk byl zaznamenán u DON testovaného v kombinaci s T-2 toxinem během 24 až 72 hodinového inkubačního času, ve srovnání s výsledky získanými jednotlivě testovaným DON. Po expozici T-2 toxinem byl zaznamenán nejsilnější inhibiční účinek na životaschopnost CHO-K1 buněk.

Zřetelný nárůst cytotoxicity byl pozorován u kombinace DON s T-2 toxinem během 24 až 72 hodinové expozice, v porovnání s výsledky získanými individuálním testováním mykotoxinů. Směsi BEA s T-2 toxinu vedou k silné redukci životnosti buněk během 24 až 72 hodinové inkubace. Můžeme tedy shrnout, že T-2 toxin vykazuje přídavný účinek na redukci životnosti buněk CHO-K1 v každém expozičním čase v kombinaci s DON a BEA.

Směsi mykotoxinů snižují buněčnou životnost v následujícím zvyšujícím se pořadí: [BEA + DON] = [T-2 toxin + DON] < [T-2 toxin + BEA].

Kombinace tří mykotoxinů významně zvyšuje cytoxické působení na buňky CHO-K1během všech expozičních časů.

The purpose of this study was to determine the capacity of three *Fusarium* mycotoxins: BEA, DON, T-2 toxin, to induce cytotoxicity using the mammalian CHO-K1 cells by the NR assay.

CHO-K1 cells were exposed to several concetrations of DON, BEA and T-2 toxin and several incubation times. The extent of cell injury was assessed by NR uptake assay, after an incubation period of 24, 48 and 72h.

Moreover, the viability of CHO-K1 cells was measured in the presence of a mixture of two or three of the mycotoxins. Significant differences were observed between the compounds tested.

All toxins, DON, BEA and T-2 toxin, tested individually diminish cell viability. Individual mycotoxins reduce viability in increasing order:

BEA<DON<T-2 toxin. Our results show that CHO-K1 cells are extremely sensitive to T-2 toxin. T-2 toxin exhibited the most cytotoxic response against the cell line tested.

Results obtained indicate that CHO-K1 cell line, exhibited a time and concentration-dependent cytotoxicity. Our results demonstrated that the T-2 toxin was found to be more cytotoxic during the exposure period, which was totally in agreement with the data previously published.

The aim of this study was also to evaluate the cytotoxicity of low concentrations of mycotoxin combinations on CHO-K1 cells. We have studied the interaction of binary or tertiary mixtures of *Fusarium* toxins on the CHO-K1 cells, by the measuring of cell viability by the NR test. All toxins, DON, BEA, T-2 toxin, tested in combination show highest decreases in cell viability compared to values obtained with individual mycotoxins.

An increase in the cytotoxicity effects was observed for BEA from 24 to 72 h of exposure when it was assayed combined with T-2 toxin, compared NR_{50} obtained alone, showing a slightly increase in cytotoxicity effects from 24 to 72 h exposition when BEA was assayed in combination with DON. DON does not show any additive effect on reduction of cell viability when it was assayed with BEA from 24 to 72h, compared to DON alone. A reduction on cell viability was showed by DON combined with T-2 toxin from 24 to 48 h, respect to NR_{50} individually obtained for DON. The highest inhibitory effect observed was after T-2 toxin exposure.

A clear increase of cytotoxicity effect was produce particularly from 24 to 72 h exposure of T-2 toxin combined with DON.Mictures of BEA and T-2 toxin lead to a strong reduction of CHO-K1 cells viability from 24 to 72 h exposure.

So, we can conclude that T-2 toxin presents additive effects in reducing viability CHO-K1 cells at any time of exposure in combination with DON and BEA.

The mixtures of mycotoxins reduce cellular viability in following increasing order: [BEA + DON] = [T-2 toxin + DON] < [T-2 toxin + BEA].

The combination of the three mycotoxins significantly increased in cytotoxicity on CHO-K1 cells in all of the time exposure.

II. INTRODUCTION

Mycotoxins are low-molecular weight natural products produced as metabolites by filamentous fungi. The Table 1 shows the main mycotoxin-producing fungal species[1].

Mycotoxins are structurally diverse compounds, which represent one the most important category of biologically produced natural toxins relative to human health and economic impact worldwide[2]. These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and agricultural products[3, 4].

Several mycotoxins, either from the same or from different fungal species, occur simultaneously in plant products[5]. Their natural co-occurrence is an increasing concern due to the hazard of exposures of humans to mixtures of mycotoxins, which could be expected to exert greater toxicity and carcinogenicity than exposure to the single mycotoxins[6].

Table 1. Fungal species and mycotoxins produced by them[7].

Mycotoxin	*Fungal species*
Aflatoxins	*Aspergilus flavus, A. parasiticus*
Beauvericin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum*
Citrinin	*Aspergilus carneus, A. terreus, Penicillium citrinum, P. hirsutum*
Deoxynivalenol	*Fusarium moniliforme, F. culmorum, F. graminearum*
Fuminosins	*Fusarium verticillioides, F. culmorum, F. avenaceum*
Nivalenol	*Fusarium moniliforme, F. equiseti, F. nivalenol*
Ochratoxin A	*Aspergilus ochraceus, Penicillium viridictum*
Patulin	*Aspergilus clavatum, Penicillium expansum*
T-2 toxin	*Fusarium acominatum, F. poae, F. sporotrichioides*
Zearalenone	*Fusarium culmorum, F. graminearum, F. oxysporum, F .roseum*

Due to diversity of mycotoxin chemical structures, they elicit a wide range of differing biological properties and toxicological effects (as is shown in Table 2), including being carcinogenic, teratogenic, mutagenic, oestrogenic, neurotoxic, or immunotoxic[2].

Table 2. Targets of mycotoxins[7].

Organ target	*mycotoxin*
Vascular system (increased vascular fragility, hemorrhage into body tissues)	Aflatoxin Satratoxin T2-toxin
Digestive system (diarrhea, vomiting, intestinal hemorrhage, liver effects, necrosis, fibrosis)	Aflatoxin T-2 toxin Deoxynivalenol
Respiratory system (respiratory distress, bleeding from lungs, pulmonary edem)	Trichothecens (T-2 toxin, Deoxynivalenol, FB1 etc.)
Nervous system (tremor, in-coordination, depression, headache)	Trichothecens (T-2 toxin, Deoxynivalenol etc.)
Cutaneous system (rash, photosensitization)	Trichothecens (T-2 toxin, Deoxynivalenol etc.)
Urinary system, nephrotoxicity	Ochratoxin A Citrinin
Reproductive system (infertility, changes in reproductive cycles)	T-2 toxin, zearalenone
Immune system (changes or suppression)	The most of mycotoxins including Beauvericin, Deoxynivalenol,T-2 toxin

III. THE AIM

The aim of this work was:

1) determination capacity of individual mycotoxins to induce cytotoxicity on the mammalian CHO-K1 cells by the measuring of cell viability by the NR test and so to confirm the risk of exposure to the mycotoxins for the human and animal health

2) determination of the cytotoxicity effects of low concentrations of mycotoxin combinations on CHO-K1 cells by the NR test and observe interactive effects of either binary or tertiary mixtures of *Fusarium* mycotoxins on the CHO-K1.

IV. THEORY

IV.1. Mycotoxins produced by Fusarium fungi

A variety of *Fusarium* fungi, which are common soil fungi, produce a number of different mycotoxins of the class of trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol) and some other toxins (zearalenone, beauvericin and fumonisins).

The *Fusarium* fungi are, probably, the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe and Asia. Most of the toxin-producing *Fusarium* species are capable of producing a variable degree two or more of these and other toxins[8].

The trichothecenes are tetracyclic sesquiterpenoid compounds with a 12,13-epoxy group. Those produced by *Fusarium* species belong to two categories according to functional groups. T-2 toxin and its metabolite, HT-2 toxin, belong to group A, which is characterized by a functional group other than a carbonyl at C-8. Trichothecenes with a carbonyl group at C-8 belong to group B. Deoxynivalenol (DON) and nivalenol (NIV) belongs to the latter group[8].

Beauvericin (BEA) is cyclic hexadepsipeptides consisting of alternating D-α-hydroxy-isovaleryl- (2-hydroxy-3-methylbutanoic acid) and amino acid -units. In BEA, the three amino acid residues are aromatic N-methyl-phenylalanines[9].

Biochemical and cellular effects and potential modes of action of trichoteeenes

The most prominent common effects of the trichothecenes T-2 toxin, HT-2 toxin, DON and NIV at the biochemical and cellular level are[8]:

- Strong inhibitory effect on the protein synthesis by binding to the ribosome
- Inhibitory effect on RNA and DNA synthesis
- Toxic effects on cell membranes.

It appears that different trichothecenes differ in their capacity to inhibit protein synthesis, to activate the mitogen activated protein kinases (MAP kinases) and to induce apoptosis. It is not clear whether the toxins work via identical mechanisms at the biochemical and cellular level.

For T-2 toxin, HT-2 toxin, DON and NIV the general toxicity and immunotoxicity are considered to be the critical effects.

Regarding myelotoxic effects T-2 toxin is the most potent both *in vivo* and *in vitro*, whereas DON shows weak toxicity towards granulocyte /monocyte progenitor cells *in vitro*.

Toxic effects of each mycotoxins are exposed belong.

T-2 and HT-2 toxins

T-2 toxin is a mycotoxin of the group trichothecenes type A produced by fungi of the *Fusarium* genus (see Table 1), which is commonly found in various cereal crops (wheat, maize, barley, oats, and rye) and processed grains (malt, beer and bread). The fungi producing trichothecenes are soil fungi and are important plant pathogens which grow on the crop in the field[10].

T-2 toxin is rapidly metabolized to HT-2 toxin, which is a major metabolite *in vivo* and therefore, a common risk assessment for T-2 toxin and HT-2 toxin appears reasonable. Some metabolites of this trichothecene are equally toxic or slightly more toxic than T-2 itself, and therefore, the metabolic fate of T-2 toxin has been of great concern. The main reactions in trichothecene metabolism are hydrolysis, hydroxylation and deep oxidation. Typical metabolites of T-2 toxin in an organism are HT-2 toxin, T- 2-triol, T-2-tetraol, 3'-hydroxy-T-2, and 3'-hydroxy-HT-2 toxin[10].

Trichothecenes are in general very stable compounds, both during storage/milling and the processing/cooking of food, and they do not degrade at high temperatures[10]. Chemical properties of T-2 toxin are shown in Table 3.

Biochemical mode of action.

T-2 toxin inhibited protein synthesis both *in vitro* (0.01 ng/ml in suspensions of rat hepatocytes gave 75 % inhibition) and *in vivo.* T-2 toxin affected the permeability of cell membranes *in vitro* at concentrations of 0.4 pg/ml, and caused changes in the phospholipid turnover in bovine platelets and haemolysis of erythrocytes *in vitro*[10].

T-2 toxin could inhibit synthesis of DNA and RNA both *in vivo* (0.75 mg/kg body weight single or multiple doses) and *in vitro* (> 0.1-1 ng/ml)[5]. T-2 toxin had a strong affinity for the 60S ribosomal subunit, and inhibited the activity of peptidyl transferase, and consequently also protein synthesis in the initiation phase[10].

A primary target of toxicity on acute toxicity is hematopoietic tissue i.e. in the bone marrow. Toxicity of the gastrointestinal epithelium is a systemic effect[10].

Acute disturbances in the circulatory system (hypotension and arrhythmia), which may result from the general pathophysiological responses to T-2 toxin including a central effect on blood pressure and catecholamine elevation, have been reported in pigs and rats[10].

General arteriosclerosis and hypertension as delayed sequela of repeated exposure to T-2 toxin have been reported. In a series of studies on rats exposed to a limited course of T-2 toxin doses caused thickening of coronary arteries including myocardial changes[10].

Chronic effect of T-2 toxin can produce hypoplasia, hyperkeratosis and acanthosis in the oesophageal region. These lesions are reversible after the exposure was terminated. High-dose males can produce pulmonary adenocarcinomas and hepatocellular adenoma.

On the other hand, acute effects of T-2 include non-specific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhea, hemorrhages and necrosis of the epithelium of stomach and intestine, bone marrow, spleen, testis and ovary, has been observed. *In vivo* inhibition of synthesis of proteins has been demonstrated in cells from bone marrow, spleen and thymus (0.75 mg/kg body weight single dose *i.p*. in mice). One single oral dose of 3.6 mg/kg body weight increased lipid peroxides in the liver of rats. However, has been observed that ascorbic acid, α-tocopherol and selenium, as well as, glutathione precursors have a protective effect against lipid peroxidation induced by T-2 toxin[10].

T-2 toxin is considered to be a major causative agent in fatal alimentary toxic aleukia in humans affecting the mucosa and the immune system. Leucopenia and necrotic lesion of the oral cavity, oesophagus and stomach are the main pathological findings[11].

Deoxynivalenol (DON)

DON is a mycotoxin produced by fungi of the *Fusarium* genus (see Table 1), which are abundant in various cereal crops (wheat, maize, barley and oats) and processed grains (malt, beer and bread). Chemically it belongs to trichothecenes. In contaminated cereals 3- and 15-acetyl DON can in significant amounts (10 – 20%) occur concomitantly with DON[12].

The substance is a very stable compound, both during storage/milling and the processing/-cooking of food and it does not degrade at high temperatures[12]. Chemical properties of DON are shown in Table 3.

Biochemical mode of action

DON inhibits the synthesis of DNA and RNA and protein synthesis at the ribosomal level. The toxin has a haemolytic effect on erythrocytes[12].

A special feature of DON toxicity is the characteristic induction of vomiting (DON is also called vomitoxin) and feed refusal seen in pigs or delayed gastric emptying and in rats and mice (weight loss and diarrhea).

The emetic effect is thought to be mediated by affecting serotonergic activity in the central nervous system (CNS) or via peripheral actions on serotonin receptors[12].

After acute intoxication necrosis in various tissues such as gastrointestinal tract, bone marrow and lymphoid tissues is also observed[12].

Zearalenone (ZEA)

ZEA is a nonsteroidal estrogenic mycotoxin produced by several *Fusarium* species (see Table 1). It is found worldwide in a number of cereal crops such as maize, barley, oats, wheat, rice and sorghum and also in bread. ZEA is a macrocyclic lactone[13].

ZEA is a stable compound, both during storage/milling and the processing/cooking of food and it does not degrade at high temperatures. Chemical properties are shown in Table 3.

Biochemical mode of action

ZEA is known for its strong oestrogenic effects, its haematotoxic and genotoxic properties. ZEA and some of its metabolites have been shown to competitively bind to estrogen receptors (ER) in a number of *in vitro* systems. Binding to specific receptors has been demonstrated in uterus, mammary gland, liver and hypothalamus from different species[13].

ZEA was shown to bind and activate both the ERα and ERβ in cells transfected with human ERα and ERβ. For ERα, ZEA was found to be a full antagonist and for ERβ to be a mixed agonist-antagonist. The relative binding affinities to the rat uterine cytoplasmatic receptor for ZEA and derivatives were α-zearalanol > α-zearalenol > β-zearalanol > ZEA > β-zearalenol[13].

This macrocyclic lactone can also induce DNA adduct formation in female mousse tissues[6].

Beauvericin (BEA)

BEA is produced by some entomo- and phytopathogenic Fusarium species (see Table 1) and other genus, such as *Beauveria bassiana*[14].

BEA is cyclic hexadepsipeptides consisting of alterating D-α-hydroxy-isovaleryl- (2-hydroxy-3-methylbutanoic acid) and amino acid -units. In BEA, the three amino acid residues are aromatic N-methyl-phenylalanines[9]. Chemical properties are shown in Table 3.

Biochemical mode of action

BEA shows antimicrobial, insecticidal, cytotoxic, ionophoric, apoptotic and immunosuppressive activity. It is the most potent specific inhibitor of cholesterol acyltransferase that is considered as toxic to several human cell lines and can induce apoptosis and DNA fragmentation[14].

BEA increases the permeability in biological membranes by forming a complex with essential cations and cation-selective channels in lipid membranes, which may affect ionic homeostasis[15].

BEA induces lipid peroxidation, has cytotoxic effect in mammalian cells and alterates kinetics in heart metabolism[16].

Fumonisins (FB)

FB are mycotoxins produced predominantly by *F. Verticillioides* (see Table 1). They are present in variable amounts in corn and corn-based feeds and food products.

Biochemical mode of action

They are suspected risk factors for esophageal cancer and neural tube defects in some human populations depending on corn as a diet staple. The most prevalent fumonisin, fumonisin B1 (FB1), causes diseases in animals associated to *F. verticillioides*, namely pulmonary edema in swine, leukoencephalomalacia in horses and cancer in the liver and kidney of rodents. Fumonisins inhibit the enzyme ceramide synthase, thereby disrupting sphingolipid metabolism, leading to accumulations of sphinganine, sphingosine and their 1-phosphate metabolites in tissues and serum and to decreases in complex sphingolipids[17].

Due to the involvement of sphigolipids in multiple physiological processes including apoptosis, mitosis, cell adhesion, pathogen recognition, and nutrient transport, it is likely that sphingolipid metabolism disruption underlies fumonisin-related diseases. This includes the induction of neural tube defects in mice, in which the uptake of folate, an essential nutrient that protects against neural tube defects, is inhibited due to a reduction in complex sphingolipids associated with lipid raft structures involved in folate transport[17].

Further studies on the mechanistic role of sphingolipids as mediators of fumonisin-induced diseases in animal models are needed to elucidate the extant to which these mycotoxins affect human health[17].

IV.2. Mechanistic interactions of mycotoxins produced by Fusarium

Several mycotoxins, either from the same or from different fungal species, occur simultaneously in plant products. However, its implication in food safety assessment is generally not known, as there is relatively little information on the interaction between concomitantly occurring mycotoxins and the consequence for the toxicity. An understanding of mode of action in simple *in vitro* stems can provide a rational basis for predicting interactions between mycotoxins[5].

Studies of combined toxic effects for exposure against mixtures of mycotoxins have been performed using animal models. Fewer studies reported the use of *in vitro* test systems for the analysis of mycotoxin mixture toxicity. Different toxicological parameters and experimental procedures were used for the analysis of the combined effects[18].

There are a few studies addressing the effects of combined exposure to several trichothecenes. Although T-2 toxin, HT-2 toxin, DON and NIV appear to cause similar effects at the biochemical and cellular level and there are similarities in toxic effects, there are also substantial differences in the spectrum of toxic effects *in vivo.* Large, non-systematic potency differences between these toxins were seen when different endpoints are considered[8].

Inhibition of protein synthesis by binding to ribosome is common to the toxins. When tested in cells *in vitro,* dose additive was observed for T-2 toxin and some other trichothecenes, whereas antagonism was observed between T-2 toxin and DON. In the protein inhibition assay, DON was hundred fold less potent than T-2 toxin and when tested at equimolar concentrations a competition for binding sites resulting in antagonism could be hypothesized. The antagonism between T-2 toxin and DON observed in the lymphocyte proliferation assay and in the yeast assay could be explained by a similar mechanism, but in neither case has this been prevent. In *in vivo* studies no synergistic interactions were observed, although each compound was administered at toxic doses[8].

In summary, dose additive, but also antagonism has been observed for T-2 toxin, DON and NIV *in vitro. In vivo* only antagonism was observed for T-2 toxin and DON and no dose additive was observed. NIV in combination with other trichothecenes has not been examined *in vivo*. Although there could be indications for dose additive in some of the *in vitro* studies, at present the database describing possible effects of combined exposure of trichothecenes is very weak and not sufficient for establishing either the nature of combined effects or the relative potencies of the trichothecenes[8].

IV.3 In vitro acute cytotoxicity tests

Previously, most of the acute toxicity data for toxins has been derived from LD_{50} bioassay of adult's mammals, that is, the amount of mycotoxin which is lethal to 50% of an exposed population after specified time interval, usually 96 hours.

In Table 4 is shown the determination of acute toxicity of chemical compounds, including mycotoxins by WHO[19].

Table 4. Classification of acute toxicity of mycotoxins according to hazard by WHO.

CLASS	Rat LD_{50}(mg/kg body weight)			
	Oral		Dermal	
	Solids	Liquids	Solids	Liquids
1a. Extremely hazardous	<5	<20	<10	<40
1b.Highly hazardous	5-50	20-200	10-100	40-400
1c.Moderately hazardous	50-500	200-2000	100-1000	400-4000
1d.Slightly hazardous	>500	>2000	>1000	>4000

Alternative methods follow the basic principles of 3 Rs (Russel and Burch, 1959) which stand for Refine, Reduce and Replace. Therefore, alternative methods are defined as the methods that Refine existing tests to minimize animal distress, reduce the animal needs for an experiment or Replace whole animal test[20].

The development of *in vitro* methods as alternatives to animal experimentation is of great relevance for the detection of the potential toxicity of mycotoxins in humans[21].

In vitro techniques have many practical advantages over *in vivo* models. The advantages of *in vitro* techniques include, rapid effective assay, control over test conditions which are not possible *in vivo*, utilization of a large number of cells per test, allowing for intrinsically continuous dose-response relationships, reduction of variability between experiments, the ability to obtain repeated samples during the course of one experiment allowing to follow the sequence of events during the toxicological response and significantly, smaller quantities of test simultaneously. Finally, and the most important advantages of *in vitro* assay is that they offer the opportunities to study and describe the mechanism of the harmful effects of chemicals[22].

Cell cultures represent alternative methods to animal experiment and allow to evaluate and to compare the toxic effects of different compounds from cytotoxicity criteria[23]. In cell culture, established cell lines derived from primary cultures by transformations processes which are spontaneous or induced are used. They may have a limited life span or continues life-span. The life span of a cell line is up, 400-500 cell cycles, and the cells are usually normal ones that are undergoing the process of senescence in vitro. They are used for basal cytotoxicity studies.

The general cellular response to a toxicological attack, in spite the great variety of mechanism and molecular targets that may be involved, can be identified in a restricted number of cellular alterations that eventually cause the death of cell.

In vitro cytotoxicity assays are useful to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions.

Cytotoxicity assays are also necessary to define the concentration range for further and more detailed *in vitro* testing to provide meaningful information on parameters such as genotoxicity or programmed cell death[24].

In vitro cytotoxicity assay are designed to evaluate the intrinsic ability of chemical to kill cells. Inhibition of cell proliferation and cell density in culture are endpoints commonly measured. Table 5 shows some of the biological endpoints for detecting cytotoxicity.

Neutral red (NR) assay is frequently used to evaluate the viability of cells. NR is a water soluble, weakly basic, survival dye that accumulates in lysosomes of viable cells[25]. Compounds that injure the plasma or lysosomal membrane decrease uptake and subsequent retention of the dye. The NR assay measures the cell membrane integrity by the incorporation of water-soluble dye into lysosomes of viable cells and is based on the spectro-photometric determination of NR taken up by viable cells and stored in their lysosomes. Cellular uptake of NR is accomplished either by pinocytosis or by passive transport across the plasma membrane. Accumulation of NR within lysosomes or from the binding of NR to fixed acidic charges, such as those of acidic polysaccharides within the lysosomal matrix. In damaged cells NR is no longer retained in cytoplasmatic vacuolar membranes and the plasma membrane does not act as a barrier to retain the dye within the cell.

Table 5. Biological endpoints for detecting cytotoxicity[26].

Endpoint	*Endpoint measurement*
Cell morphology	Cell size and shape Cell-cell contacts Nuclear size, shape and inclusion Nuclear vacuole formation Cytoplasmatic vacuole formation
Cell viability	Vital dye uptake Trypan blue exclusion Cell number Replacing efficiency
Cell adhesion	Attachment to culture surface Cell-cell adhesion
Cell proliferation	Increase in cell number Increase in total DNA Increase in total RNA
Membrana demage	Loss of enzyme Loss of ions cofactors Leakage across cellular membrane Leakage from pre-loaded cells

V. EXPERIMENTAL

V.1 Chemicals and materials

Materials and mycotoxins

The reagent grade chemical dimethylsulfoxide (DMSO) and cell culture components used, namely nutrient mixture Ham F-12, penicillin-streptomycin (Gibco BRL®), trypsin/EDTA solution (Sigma-cell culture™), (N-[2-hydroxyethyl]piperazine-N'-[zethanesulfonic acid)] (HEPES), 3-amino-7-dimethylammino-2-methylphenazine hydrochloride (neutral red dye), trypan blue, acetic acid, ethanol, methanol, $CaCl_2$, ethylenediaminetetraacetic acid (EDTA) and formaldehyde 37-40 % were obtained from Sigma Chemical Co. (St. Louis Mo. USA). Fetal bovine serum (FBS) was from Cambrex Company (Belgium).

Mycotoxins, DON, T-2 toxin, BEA with purity up to 99% were supplied by Sigma Chemical Company (St. Louis, MO, USA). Chemical structure and molecular weights of all the mycotoxins studied are shown in Table 3. The primary stock solution of each mycotoxins was completely dissolved in distilled water or sterilized methanol (when it was not possible in water).

Equipments

- Incubator THERMO SCIENTIFIC HEPA CLASS 100, model 371,USA
- Microscope NIKON eclipse TE2000-S, Japan
- Beckman Coulter Z1 particle counter, Germany.
- Centrifuge 5810R eppendorfs AG, Hamburg
- Laminar Cabin: TELSTAR BIO-II-A, Valtek Nova, Spain
- Microtiter plate reader Wallace Victor, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland.
- Analytical weight METTER AJ100L (δ=0,0001), Spain

V.2. Cell culture

V.2.1 Cell lines and maintenance

The Chinese Hamster Ovary (CHO-K1) cell line from *Circetulus griseus* was obtained from American Type Culture Collection (ATCC CCL 61). CHO-K1 cells were grown in polystyrene tissue culture dishes at 37ºC, in a humidified 5% CO_2 atmosphere in Ham-F12 supplemented with 10% FCS and 100 U/ml penicillin and 100µg/ml streptomycin.

Cells were subculture as they reached confluence. For this process the medium was removed and the cells were gently washed two times with 5 ml of medium or PBS. The cells were dissociated by addition of 1 ml of trypsin (0,005%)/EDTA (0,002%). A few seconds of exposure removed the cells, longer results in cell death. Six ml of culture medium was added to each culture dishes and homogenized gently to disrupt cell clumps. The cells were then either passed directly to new flasks (usually 1:3) or counted the number of cells per ml with a Beckman particle counter and then seeded to each of the wells of a 96 well plate. All these procedures were performed under sterile conditions; manipulations were carried out in a laminar flow hood.

V.2.2 Cell viability

Before the cells were cultured their viability and number were determined with a haemocytometer using trypan blue stain. The method is based on the principle that live cells do not take up vital dyes such as trypan blue, due to the intact plasma membrane excluding them, whereas dye penetrates dead cells staining the nucleus.

100 µl trypan blue was added to 100 µl of cell suspension. A cover slip was placed on the haemocytometer and the chambers were filled using capillary action by touching the edge of the cover slip with an autopippette containing the dye/cell suspension. Both viable and non-viable cells were counted in the 1 mm centre square.

V.2.3 Cytotoxicity assay

The cytotoxicity of individual and combined mycotoxins was estimated in CHO-K1 cells using the NR assay. The NR assay method was performed and described by Ruiz et al. (2006)[19]. CHO-K1 cells were harvested in 96-well tissue culture microtiter plate at a density of 2 x 10^4 cell/well in Ham-F12 medium with 10% FCS. Cells in 200µl medium were seeded to each of 96 wells in a microtriter plate to form a monolayer of about 65% of confluence. It is important that confluence has not been reached at the time of testing so that cells are fully exposed to toxic agents during cell division, since confluent monolayer cultures are less sensitive to toxic agents.

After the cells reached 65% confluence, the medium was removed and replaced with 200 µl of fresh medium containing decanally increasing concentrations of mycotoxins. After preliminary studies, test concentration was selected from 0.5 to 50 µM for BEA and DON, and from 3 to 300 nM for T-2 toxin. Normally at least 8 control wells were used in each experiment. Each concentration tested, medium was distributed over 8 wells. All additions to the cells were performed with care, using an Eppendorf pipette, to avoid detaching the cells. During all the experiments the cells were scanned under a light microscope to check for bacterial/fungal contamination.

After the plates were incubated at 37 °C for 24, 48 and 72 hours the medium with mycotoxins, was replaced with fresh medium containing 50µg/ml NR dye. The fresh medium containing NR had previously been incubated overnight at 37°C and was filtered, using a 0.25 µm filter, before addition to remove fine precipitation and dye crystals formed. Deposit of such precipitated crystals into the cell cultures during incubation would interfere with the assay.

Once, NR dye was added to cells, the microtiter plate was returned to the incubator for another 3 hours to allow for the uptake of the supravital dye into lysosomes of viable, uninjured cells. After this period the plates were again scanned, by light microscope to ensure no crystals had formed.

After 3 h, cells were washed 200µl of a solution of 0.5% (v/v) formaldehyde and 1% $CaCl_2$ to remove unincorporated dye and simultaneously promote adhesion of the cells to the substratum. The formaldehyde was left only briefly in contact with the cells (10 seconds), since longer exposure would result in extraction of the dye. After removal, cells were fished with 200µl of a solution of 1% acetic acid in 50% ethanol. After approximately 30 minutes at incubator, the 96 well plates were rapidly agitated for a five minute on a microtiter plate shaker and NR was quantified using a microtiter plate reader (Wallace Victor, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) at 490 nm.

V.2.4 Statistical analysis of data

To evaluate the cytotoxicity of the tested compounds, seven different concentrations of each mycotoxin were tested. All experiments were performed at least three times, in separate days.

The NR_{50} values, defined as the concentration of test mycotoxins that modified the cell viability by 50% in comparison with the appropriate untreated control cultures, were calculated from the dose-response curve. The results thus obtained are expressed in the Fig. 2 to 8, as percentage change compared with unexposed cells. NR_{50} values were determined by linear interpolation. Statistical analysis was performed using analysis of variance (ANOVA) and a comparison of means with a Student's t-test (two tails) was applied out to determine the differences between cytotoxicity by CHO-K1 cells after incubation with different mycotoxin amounts. Data were expressed as means ± SD from at least three experiments. *P*-values<0.05 were considered statistically significant. Slopes and regression coefficients were estimated from each toxicity regression line using the Sigma Plot program.

VI. RESULTS AND DISCUSSION

The present study was undertaken to evaluate the capacity of three mycotoxins, BEA, DON and T-2 toxin, to induce cytotoxicity using the mammalian CHO-K1 cells by the NR assay. CHO-K1 cells were exposed to several concentrations of DON, BEA and T-2 toxin and several incubation times. The extent of cell injury was assessed by NR uptake assay, after an incubation period of 24, 48 and 72h. NR was used similarly by other authors to determine the cytotoxic effects of trichothecene toxins[19]. Moreover, the viability of CHO-K1 cells was measured in the presence of a mixture of two o three of the mycotoxins.

Figures 1 and 2 show CHO-K1 cells previously, and during combination of mycotoxins exposition.

Figure 1. CHO-K1 cells observed at microscopy without mycotoxin exposition at, a) 100% of cell confluence and b) 65% of cell confluence.

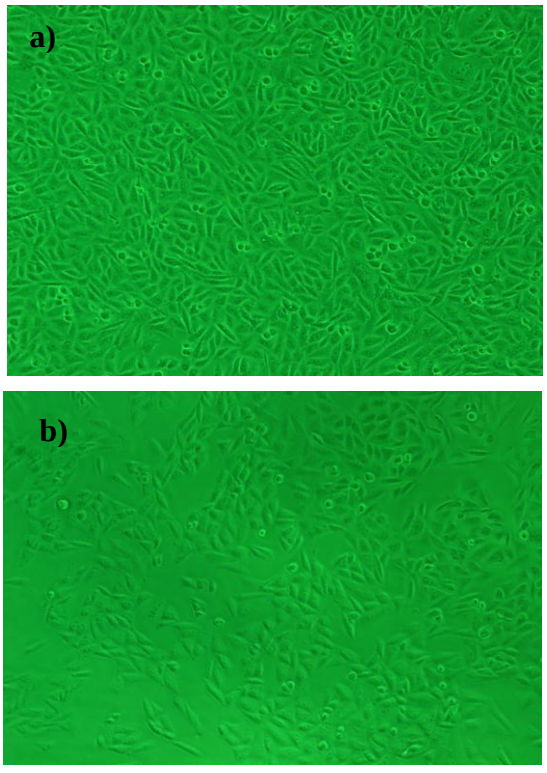
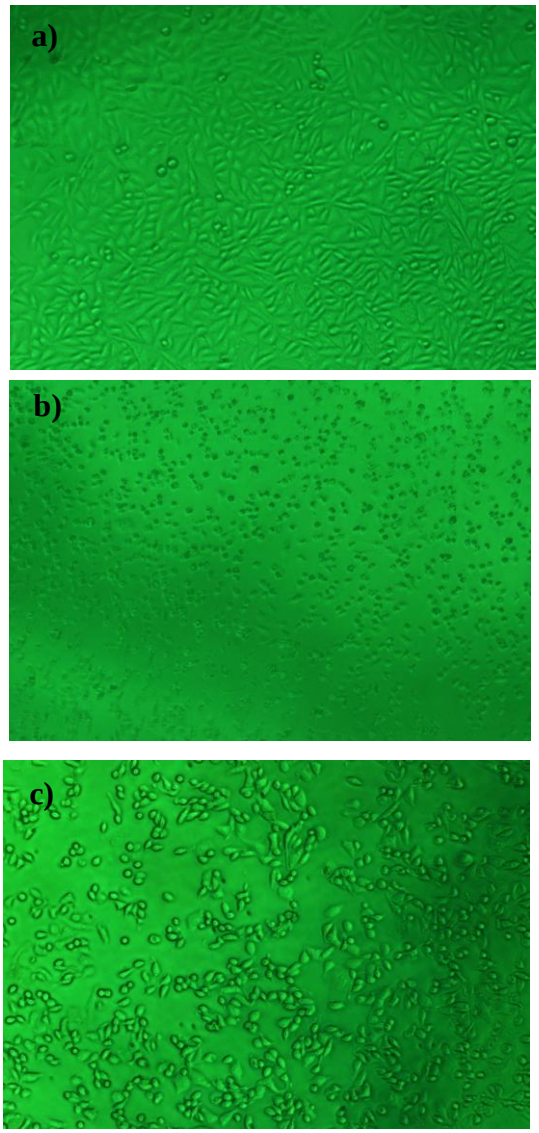


Figure 2. Cytotoxic effect of combination of two mycotoxins on CHO-K1 cells, observed at microscopy, after 24 h incubation evaluated by NR test of the binary mixtures of: a) 1 µM DON and 3.125 µM BEA; b) 20 µM DON and 50 µM BEA; and c) 300 nM T-2 toxin and 10 µM DON.



VI.1. Influence of individual mycotoxins on CHO-K1 cell viability

Cellular viability was conduced on CHO-K1 in a 96-well microtiter plates. The 65% confluent monolayer CHO-K1 cells were exposed to mycotoxins at the different concentration range for each mycotoxin and incubation time.

Table 6 shows the NR_{50} of BEA, DON and T-2 toxin on CHO-K1 cells after

24h, 48h and 72h exposure using the NR assay.

Figures 3 to 5 show the concentration-response curves for the individual mycotoxins, at 24, 48, and 72 h. Significant differences were observed between the compounds tested. All toxins, DON, BEA, T-2 toxin, tested individually diminish cell viability (Table 6).

Fig 3: EC_{50} (µM) on CHO-K1 cells of the exposure to BEA for 2, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values (P < 0.05).

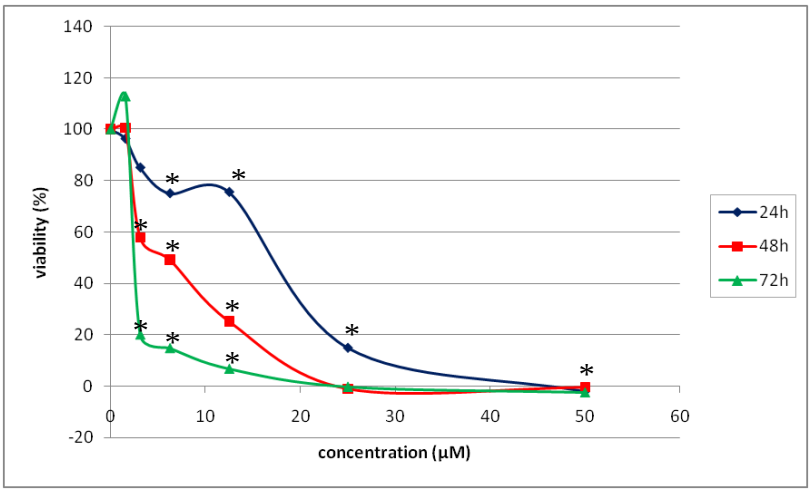


Fig 4: EC_{50} (µM) on CHO-K1 cells of the exposure to DON for 24, 48, 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values (P < 0.05).

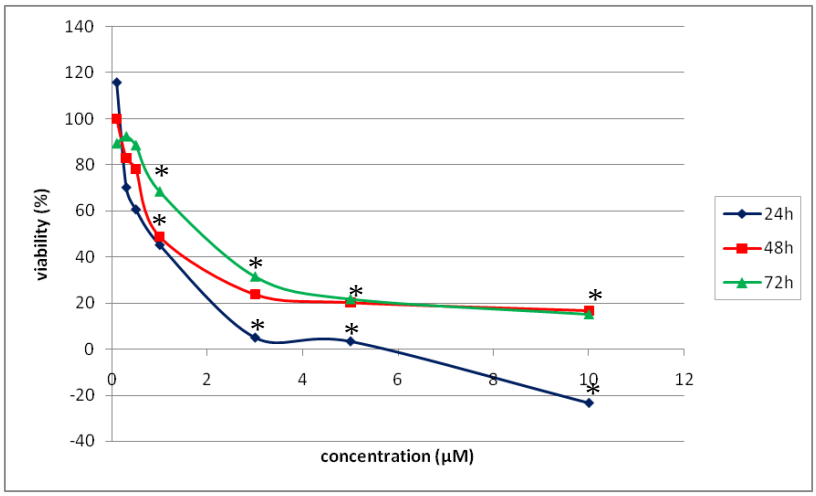
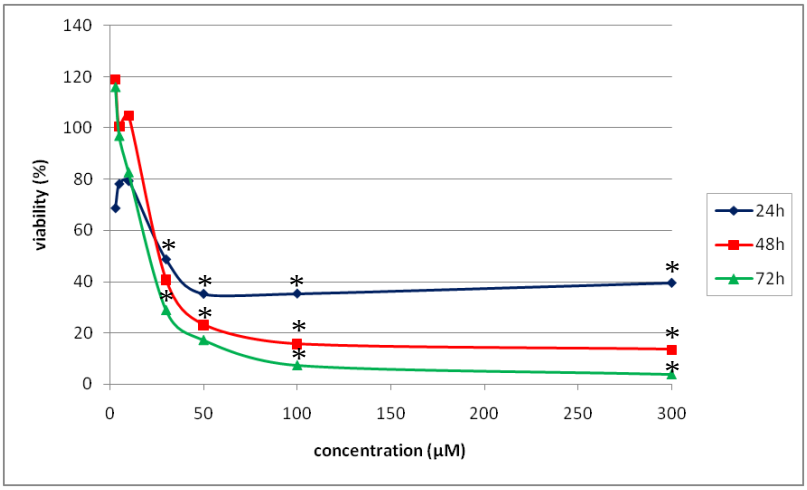


Fig 5: EC_{50} (nM) of CHO-K1 cells of the exposure to T-2 toxin for 24, 48, 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).



Individual mycotoxins reduce cellular viability in increasing order: BEA<DON<T-2 toxin. The cytotoxicity of mycotoxins tested show a range from high to relative low when compared to each other with T-2 toxin, being at least 95 times more toxic that BEA on CHO-K1 cells. Our results, demonstrated that CHO-K1 cells are extremely sensitive to T-2 toxin (Table 6). T-2 toxin exhibited the most cytotoxic response against the cell line tested. Results obtained indicate that CHO-K1 cell line, exhibited a time and concentration-dependent cytotoxicity (as show in Table 6).

Table 6. NR_{50} cytotoxicity values of the individual mycotoxins tested after 24, 48 and 72h exposure, on CHO-K1 cells.

Mycotoxin	*Cytotoxicity (NR_{50}, µM)*		
	24 h	**48 h**	**72 h**
Beauvericin	8.20 ± 0.74	6.20 ± 0.06	3.80 ± 1.18
Deoxynivalenol	2.30 ± 0.46	1.94 ± 0.52	1.83 ± 0.02
T-2 toxin	52.19 x 10^{-3} ± 8.24	50.27 x 10^{-3} ± 13.43	41.72 x 10^{-3} ± 0.72

The cytotoxicity of mycotoxins DON, BEA and T-2 toxin, has been studied in cell lines of different origin by several authors and the mean effective concentration values were different as different sensitivities of them. However, remarkable differences between studies of the mean effective concentrations for identical cell lines were reported, independent of the chosen parameter of cytotoxicity. This fact corroborates that differences in specific cytotoxicity can be attributed to the use of different cell lines, different endpoints, presence or absence of serum in the culture medium, incubation time, combinations of toxins and concentrations ranging assayed (Table 7).

Table 7. Cytotoxicity values of EC_{50} of individual mycotoxins reported by literature. Incubation time is expressed in brackets.

Mycotoxins	*Cells*	*Assay*	*EC50*	*Reference*
BEA	SF-9	MTT	2.5 μM (48h)	27
	U-937	Trypan blue	30 μM (24 h)	28
	HL-60		15 μM (24 h)	
DON	Caco-2	NR	21.5 μM (72h)	31
		MTT	25 μM (72h)	
	CHO-K1	MTT	0.27 μg/ml(48h)	29
	Caco-2		1.02 μg/ml (48h)	
	C5-O		0.54 μg/ml (48h)	
	V79		0.49 μg/ml (48h)	
	HepG-2		8.36 μg/ml (48h)	
	Caco-2	NR	21.5 μM (72 h)	6
T-2 toxin	Vero	NR	4 nM (24h)	26
		MTT	60 nM (24h)	
	Vero	MTT	110 nM (24 h)	30
	HGF-1	NR	0.25 ng/ml (72h)	31
	SW 742	NR	5.5 ng/ml (72h)	
	HepG-2	NR	2 ng/ml (72h)	

NOTE: SF-9 (the insect cells from *Spodoptera frugiperda*); U-937 (monocytic lymphoma cells); HL-60 (promyelocytic leukemia cells); Caco-2 (human adenoma colon cell line); C5-O (Balb/c mice keratinocyte cell line); V79 (chinese hamster lung fibroblast cell line); HepG-2 (hepatocellular carcinoma cell line); Vero (kidney cells from *Cercopithecus aethiops*); HGF-1 (gingival cells from humans); SW 742 (human colon carcinoma cells); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium).

Our results demonstrated that the T-2 toxin was found to be more cytotoxic during the exposure period, which was totally in agreement with the data previously published (table 7).

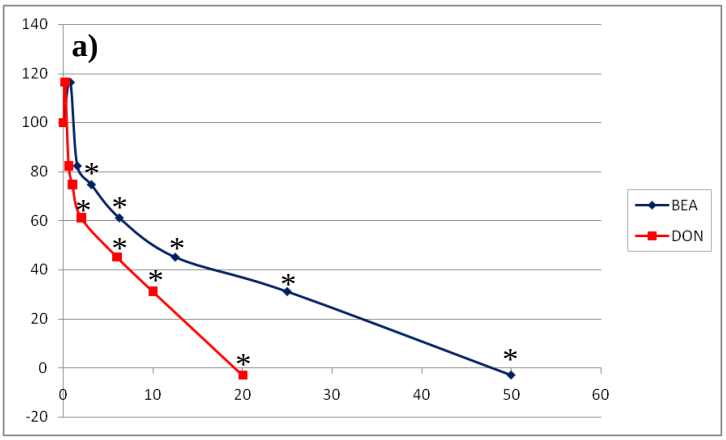
VI.2 Influence of mixtures of mycotoxins on CHO-K1 cell viability

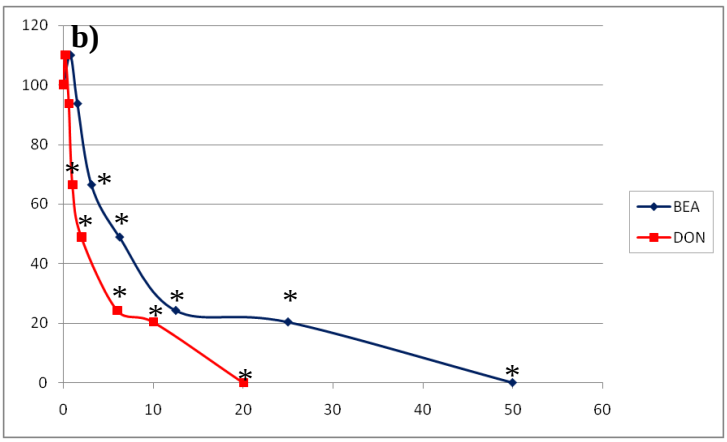
Because natural co-occurence of BEA, DON and T-2 toxin in maize is rather common, the purpose of this study was to determine the cytotoxic effects of relatively low concentrations of mycotoxin combinations on CHO-K1 cells. The interactive effects of either binary or tertiary mixtures of *Fusarium* mycotoxins, DON, BEA and T-2 toxin on the CHO-K1 cells, by the measuring of cell viability by the NR test have been studied.

Figures 6 to 8 show the concentration-response curves for the mixture of mycotoxins, at 24, 48, and 72 h. Significant differences were observed between the compounds tested. All toxins, DON, BEA, T-2 toxin, tested in combination show highest decreases in cell viability compared to values obtained with individual mycotoxins tested (Table 8).

When compared mean neutral red concentration obtained with combined mycotoxins (Table 8) and those obtained with mycotoxins alone (Table 6) different response was observed depending on the mycotoxins combined and the exposure time. An increase in cytoxicity was observed for BEA from 24 to 72 h of exposure when it was assayed combined with T-2 toxin, compared NR_{50} obtained alone; showing comparable effects from 24 to 72 h exposition when BEA was assayed in combination with DON. On the other hand, DON does not show any additive effect on reduction of cell viability when it was assayed with BEA from 24 to 72 h, compared to DON assayed alone. However, slightly reduction on cell viability was showed by DON combined with T-2 toxin from 24 to 48 h, respect to NR_{50} individually obtained for DON.

Fig 6. EC_{50} (nM) of CHO-K1 cells of the exposure to BEA and DON for a) 24 h, b) 48 h, and c) 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).





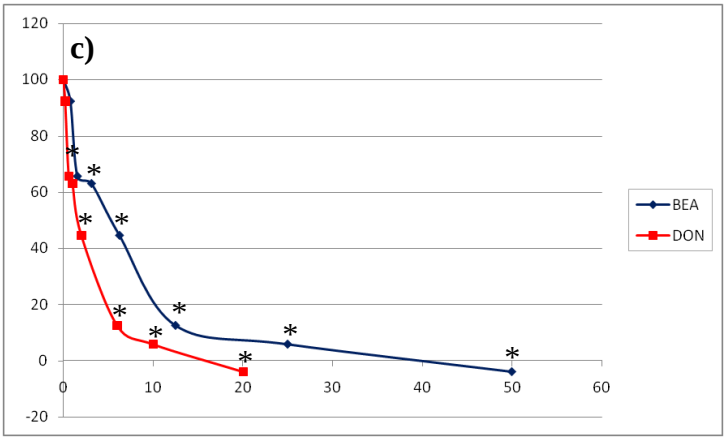
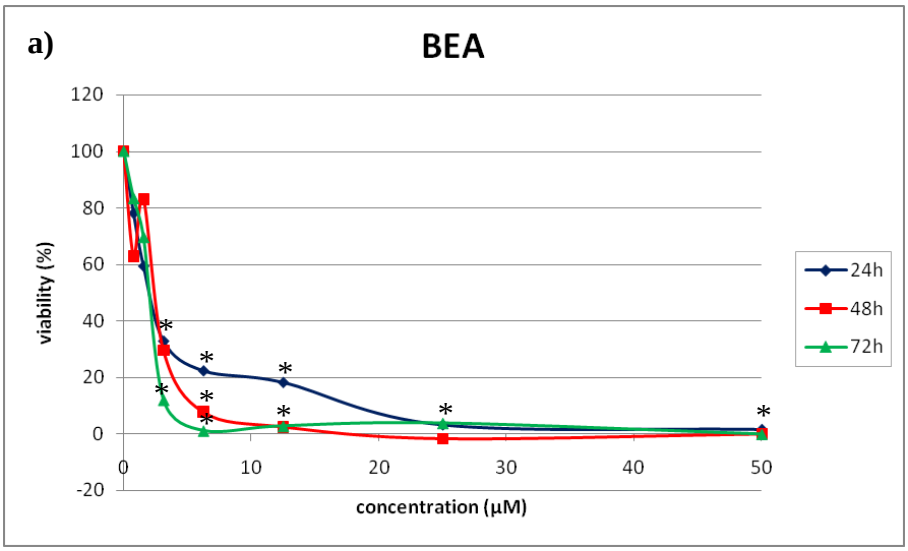


Fig 7. EC_{50} (nM) of CHO-K1 cells of the exposure of a combination of a) BEA and b) T-2 toxin for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).



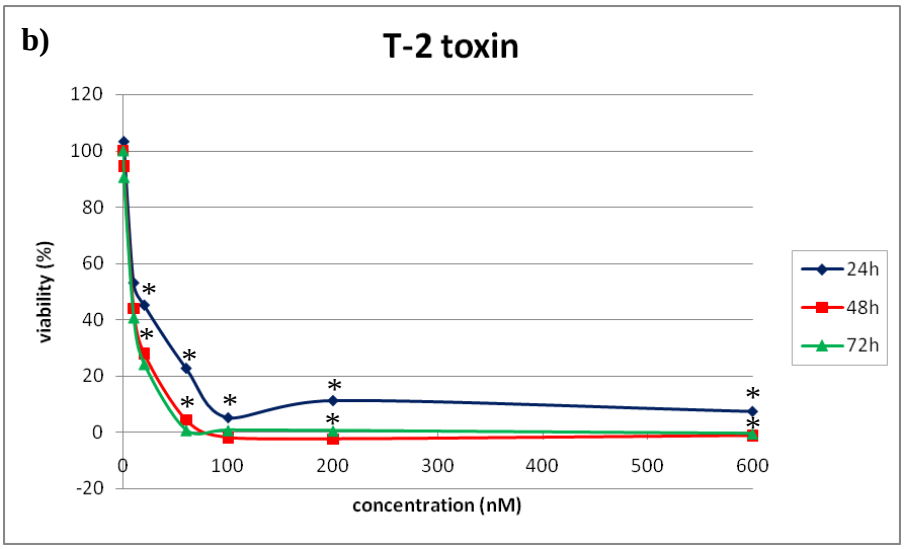
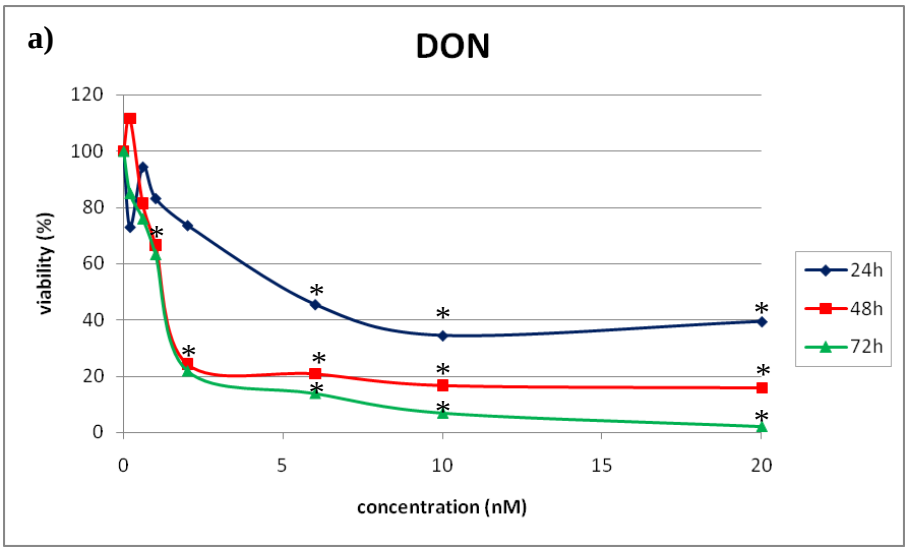
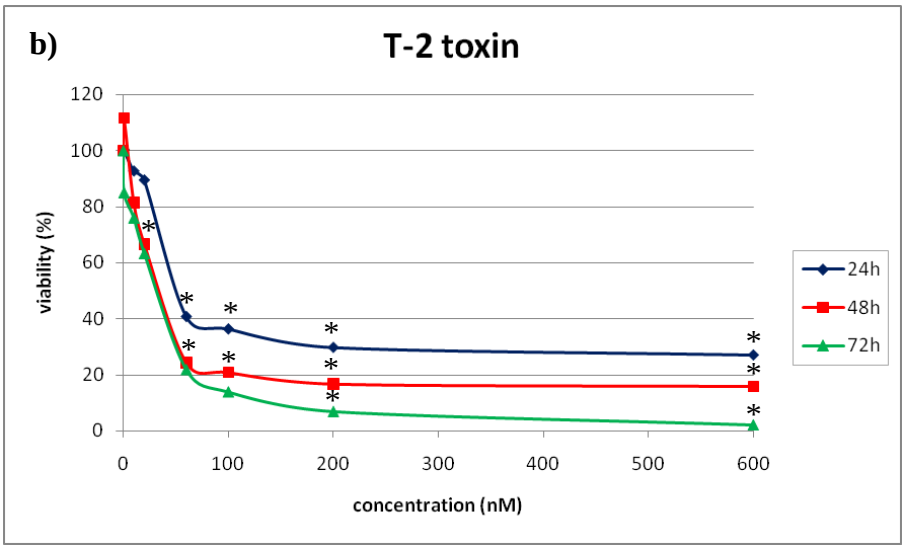


Fig 8. EC_{50} (nM) of CHO-K1 cells of the exposure of a combination of a) DON and b) T-2 toxin for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).





The highest inhibitory effect observed was after T-2 toxin exposure. A clear increase of cytotoxicity effect was produce particularly from 48 to 72 h exposure of T-2 toxin combined with DON. Mixtures of BEA and T-2 toxin lead to a strong reduction of CHO-K1 cells viability from 24 to 72 h exposure (Table 8). So, we can conclude that T-2 toxin presents additive effect in reducing viability CHO-K1 cells at any time of exposure in combination with DON and BEA.

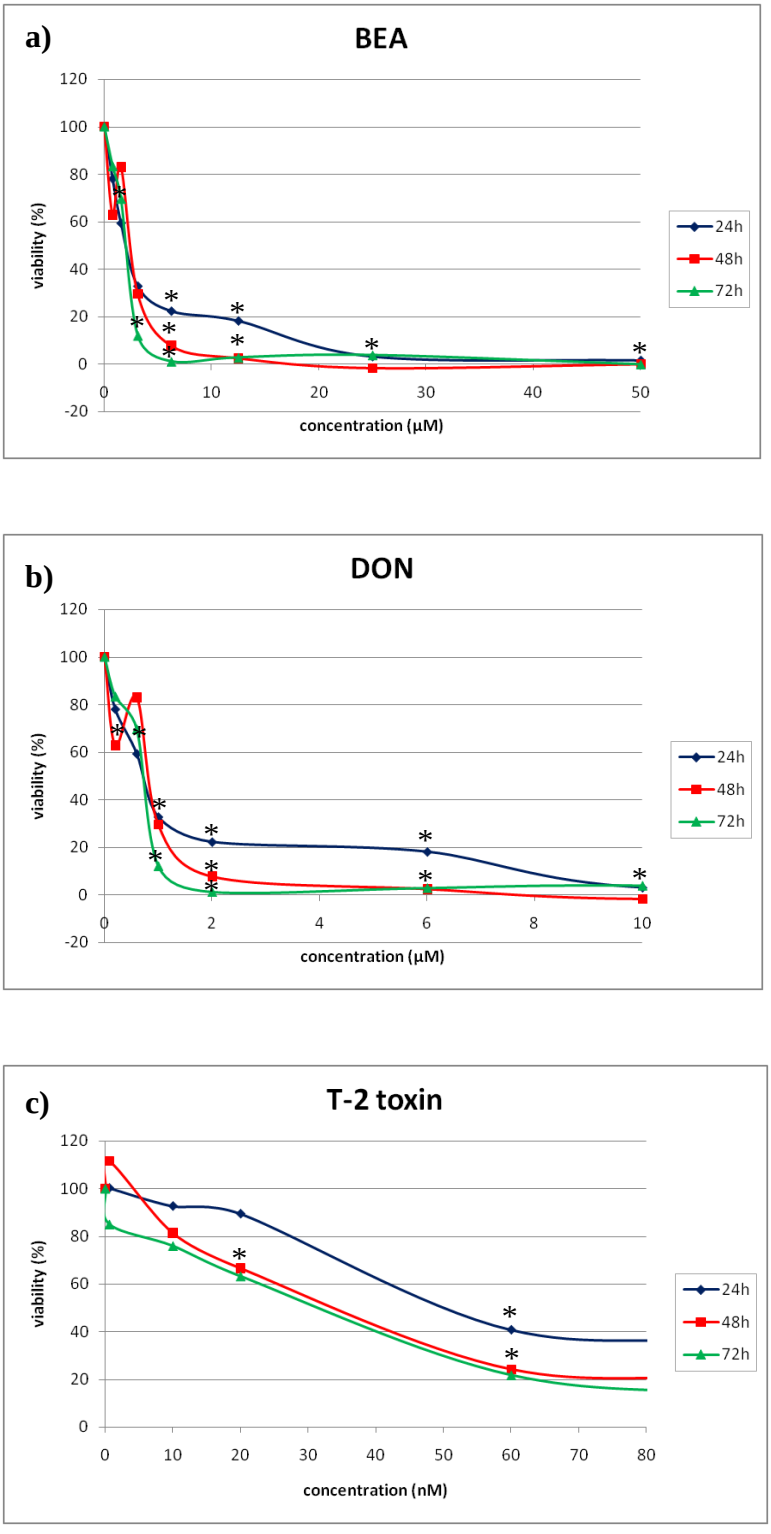
Table 8. NR_{50} cytotoxicity values of the combination of mycotoxins tested after 24, 48 and 72 h exposure, on CHO-K1 cells.

Mycotoxin	*Cytotoxicity (NR_{50})*					
BEA+DON	**BEA (µM)**			**DON (µM)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	7.00 ± 0.72	6.46 ± 0.04	3.37 ± 1.16	2.53 ± 0.64	2.31 ± 0.25	1.15 ± 0.01
T-2+DON	**T-2 toxin (nM)**			**DON (µM)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	49.00 ± 4.82	35.00 ± 4.31	31.6 ± 2.07	1.59 ± 0.02	1.22 ± 0.02	1.17 ± 0.01
T-2+BEA	**T-2 toxin (nM)**			**BEA (µM)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	11.84 ± 2.54	8.70 ± 2.31	7.39 ± 3.10	3.42 ± 0.27	2.89 ± 0.01	2.70 ± 0.18

The mixtures of mycotoxins reduce cellular viability in following increasing order: [BEA + DON] = [T-2 toxin + DON] < [T-2 toxin + BEA].

The mean NR concentration obtained from the mixture of the three mycotoxins (DON, BEA and T-2 toxin) at 24, 48 and 72 h exposure is shown in Table 9. Figure 9 shows the concentration-response curves for the three mycotoxins, at 24, 48, and 72 h tested together. Significant differences were observed between the compounds tested. All toxins, DON, BEA, T-2 toxin, highest increase in diminish cell viability compared to individual mycotoxins tested (Table 9).

Fig 9. EC_{50} (nM) of CHO-K1 cells of the exposure of a combination of BEA and DON for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).



The combination of the three mycotoxins significantly increased in cytotoxicity on CHO-K1 cells in all of the time exposure. This effect was even more pronounced to the same concentration with the mixtures of two mycotoxins tested, independently of those two combined (Tables 8 and 9).

Table 9. NR_{50}Cytotoxicity values of the combination of the three mycotoxins tested, BEA, DON and T-2 toxin, after 24, 48 and 72 h exposure on CHO-K1 cells.

Mycotoxin	*Cytotoxicity (NR_{50}; µM)*		
	24h	**48h**	**72h**
Beauvericin	2.17 ± 0.01	1.85 ± 0.02	1.67 ± 0.03
Deoxynivalenol	0.69 ± 0.01	0.57 ± 0.00	0.51 ± 0.00
T-2 toxin	$3.22x10^{-3}$ ± 0.03	$2.69x10^{-3}$ ± 0.01	$2.33x10^{-3}$ ± 0.00

Although cytotoxic effects of individual mycotoxins have been studied; limited information exists in literature about synergism, addition and antagonism effects against cells exposed to mixture of mycotoxins. Data obtained from literature differ from those of this study particularly in the type of mycotoxins used in the mixtures. There are a few studies addressing the effects of combined exposure to several trichothecenes. Kouadio et al (2007) studied the interactive cytotoxicity effects of either binary or tertiary mixtures of *Fusarium* mycotoxins, DON, ZEA and FB1 on the intestinal cell line, Caco-2 using the NR assay[6]. They found that the mixtures of mycotoxins reduce cellular viability in the following increasing order: [FB1 + ZEA] < [FB1 + DON] < [ZEA + DON] < [FB1 + DON + ZEA]. Moreover, they observed that FB1 antagonizes the effects of ZEA, thus mixture of FB1 and ZEA improves Caco-2 cells viability in contrast to individual effects. On the contrary, mixtures of ZEA or FB1 and DON, display synergistic effects.

Other studies, related to cytotoxicity effects of mycotoxins were observed in literature. These studies, the great interest with regard to human and animal health, have not been commented here, because they were related to combined mycotoxins produced by a number of *Penicillium*, *Aspergillus* and other fungus genus, different from *Fusarium*.

Although T-2 toxin, HT-2 toxin, DON and NIV appear to cause similar effects at the biochemical and cellular level and there are similarities in toxic effects; however there are also substantial differences in the spectrum of toxic effects *in vivo*[8]. When tested in cells *in vitro,* dose additive was observed for T-2 toxin and some other trichothecenes, whereas antagonism was observed between T-2 toxin and DON. In the protein inhibition assay DON was hundred fold less potent than T-2 toxin and when tested at equimolar concentrations a competition for binding sites resulting in antagonism could be hypothesised. The antagonism between T-2 toxin and DON observed in the lymphocyte proliferation assay and in the yeast assay could be explained by a similar mechanism, but in neither case has this been prevent.

In conclusion, the results of the three mycotoxins assayed in CHO-K1 cells demonstrate that the NR assay can provide useful information of toxic effects of mycotoxins at the cellular level. Moreover, the point of view of mycotoxin-related health risk, of greatest concern is the co-occurrence and combination of these mycotoxins in foodstuffs, e.g. grain and cereals, and their possible synergistic interactions might be an important trigger for development of acute and/or chronic toxicity in consumers, especially after long-term exposure.

VII. CONCLUSIONS

1.All individual tested mycotoxins (DON, BEA, T-2) diminish cell viability by the NR test. The degree of cell survival is dependent on concentration of toxin and period of exposure.

2. Individual mycotoxins reduce cellular viability in increasing order: BEA<DON<T-2 toxin. The CHO-K1 cells are extremelly sensitive to T-2 toxin.Our results demonstrated that the T-2 toxin was found to be more cytotoxic during the exposure period, which was totally in agreement with the data previously published.

3. The mixtures of DON and BEA show higher reduction of cell viability in NR test, as compared to DON or BEA alone.

4. The strong inhibion of cell viability was observed after the exposure to the mixtures of BEA+T-2 and DON+T-2 toxin.
T-2 toxin presents additive effect in reducing cell viability of CHO-K1 cells in combination with DON and BEA at any time of exposure.

5. The mixture of the three mycotoxins (DON, BEA and T-2 toxin) significantly increased cytotoxicity on CHO-K1 cells in all time exposure.

6. The mixtures of mycotoxins reduce cellular viability in increasing order: [BEA+DON]<[T-2+DON]<[T-2+BEA]<[BEA+T-2+DON].

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