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Pro-apoptotic effects of nivalenol and deoxynivalenol trichothecenes in J774A.1 murine macrophages

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ABSTRACT

Nivalenol (NIV) and deoxynivalenol (DON) are trichothecenes mycotoxins produced by *Fusarium* fungi that occur in cereal grains alone or in combination. Several studies have shown that exposure to high concentrations of these mycotoxins resulted in decreased cell proliferation; however, the molecular mechanism underlying their activities are still partially known.

In this study, we evaluated the effects of NIV and DON, alone and in combination, on J7741.A macrophages viability. The results of the current study show that both NIV and DON (10–100 μM) significantly stimulate apoptosis in J774A.1 macrophages in a concentration-dependent manner; in particular, NIV results a stronger pro-apoptotic effect than DON on cultured J774A.1 murine macrophages. No interactive effects were observed by exposing J774A.1 cells to both NIV and DON simultaneously. Pro-apoptotic activity induced by both mycotoxins seems to be essentially mediated by caspase-3 and is associated with a cell cycle blocking in G0/G1 phase. Moreover, our results show that NIV and DON are able to influence apoptotic pathway by ERK, pro-apoptotic protein Bax, caspase-3 and poly-ADP-ribose synthase (PARP), DNA repairing enzyme.

1. Introduction

Mycotoxins are food contaminants produced by secondary metabolism of fungi found primarily in cereal grains and derived products. They are heterogeneous group of chemicals that elicit different toxic effects both on human and animal health. More than 400 different mycotoxins have been isolated and chemically characterized; those of major medical and agricultural concern are aflatoxins, ochratoxins, trichothecenes, zearalenone and fumonisins. Food contamination by mycotoxins assumed great importance in recent years for their impact on human and animal health and because they are very frequently found in food and feed in particular in cereals such as wheat, maize, barley, oats, and rye but also in cocoa, spices, wine and many other products and processed grains. Data of FAO show that about 25% of world food production is contaminated by at least one mycotoxin (Heussner et al., 2006). Trichothecenes mycotoxins are chemically related compounds produced by different fungal genera, including *Fusarium*, *Mycothecium*, *Trichoderma*, *Trichothecium*, *Stachybotrys*, *Verticimonosporium*, and *Cephalosporium* (Ueno, 1985).

A data collection on the occurrence of *Fusarium* toxins in food in the European Union showed a 57% incidence of positive samples for deoxynivalenol (DON) and 16% for nivalenol (NIV), out of several thousands of samples analysed (Schothorst and Van Egmond, 2004); moreover, these mycotoxins are very stable both during storage/milling and processing/cooking of food (Bretz et al., 2006; Hazel and Patel, 2004).

Food and feed commodities are usually contaminated by different mycotoxins concomitantly, often produced by the same mold species. However, there is relatively little information about the interaction between co-occurring mycotoxins and the consequences for their toxic effects (Speijers and Speijers, 2004). Only few studies focused on the combined administration of trichothecenes in cell systems *in vitro* showing that their co-occurrence exerts interactive (Tajima et al., 2002) or no interactive effects in different cell systems (Luongo et al., 2008).

Trichothecenes are harmful to human and animal health causing a range of acute and chronic symptoms (D'Mello and Mac Donald, 1997); these mycotoxins are able to bind to eukaryotic ribosomes and inhibit protein synthesis by blocking translation and inhibiting the elongation of the peptide chain. Different toxic properties have been associated with trichothecenes; acute and subacute exposure

Abbreviations: DON, deoxynivalenol; NIV, nivalenol; MAPK, mitogen-activated protein kinases; PARP, poly-ADP-ribose polymerase; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; OD, optical density; PI, propidium iodide; PBS, phosphate buffered saline; s.e.m., standard error of the mean.

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to high doses of DON is characterized by emetic response, anorexia, diarrhoea, leukocytosis, haemorrhages and circulatory shock in swine, dog and humans whereas chronic exposure affects growth, reproduction and immune functions in animals (Larsen et al., 2004). Some studies with experimental animals demonstrated effects on immune system, including impaired delayed-type hypersensitivity responses, phagocyte activity (Pestka et al., 1994; Rotter et al., 1996) and modulation of host response to enteric infections (Li et al., 2005). Both *in vivo* and *in vitro* studies showed that trichothecenes can suppress or enhance immune functions (Bondy and Pestka, 2000). In particular, NIV inhibits blastogenesis in cultured human lymphocytes (Forsell and Pestka, 1985) and total and antigen specific IgE production in ovoalbumin specific T-cell receptor αβ transgenic mice (Choi et al., 2000). Trichothecenes have the potential to both directly induce and superinduce pro-inflammatory cytokine and chemokine expression in human macrophages, even at concentrations that are cytotoxic (Sugita-Konishi and Pestka, 2001). Macrophages and innate immune response appear to be exquisitely sensitive to trichothecenes that up-regulate expression of inflammation-related genes *in vivo* and *in vitro* at low concentrations including COX-2 (Moon and Pestka, 2003), proinflammatory cytokines (Wong et al., 1998; Zhou et al., 1997), nitric oxide synthase (Ji et al., 1998), and numerous chemokines (Chung et al., 2003; Kinser et al., 2004). In contrast, exposure to high concentrations of trichothecenes is able to induce apoptosis in macrophages (Yang et al., 2000; Zhou et al., 2003) thereby suppressing innate immune function (Pestka et al., 2004). In particular Zhou et al. (2005) have shown the capability of DON to induce apoptosis in murine macrophages through the activation of several mitogen-activated protein kinases (MAPKs).

While several studies regarding the effects of DON on macrophage functions have been carried out, few are known about the effects of NIV and the mixture of the two trichothecenes on these cells. In this study, we report the effects of NIV on macrophage cell viability and immune response comparing these effects with those of DON and evaluated the effects of co-exposure of NIV and DON simultaneously in J774A.1 cultured macrophages.

2. Materials and methods

2.1. Reagents

Unless stated otherwise, all reagents and compounds were obtained from Sigma Chemicals Company (Sigma, Milan, Italy).

2.2. Anti-proliferative activity

J774A.1, murine monocyte macrophage cell line (American Type Culture Collection, Rockville, MD), was grown in adhesion on Petri dishes and maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mM HEPES, 2 mM glutamine, 100 u/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

Cells (3.4×10^4) were plated on 96-well microtiter plates and allowed to adhere for 2 h. Thereafter, the medium was replaced with 90 μL of fresh medium and a 10 μL aliquot of serial dilutions of NIV and DON (10–100 μM), alone and in binary combination (1:1 ratio), was added. Cells were incubated for 24, 48 and 72 h. Cell viability was assessed through MTT assay (Mosmann, 1983). Briefly, 25 μL of MTT (5 mg/mL) were added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 μL of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. Macrophage viability in response to treatment with NIV and DON, alone and in combination, was calculated as: % dead cells = 100 − (OD treated/OD control) × 100.

2.3. Analysis of apoptosis

Hypodiploid DNA was analysed using propidium iodide (PI) staining by flow cytometry. Briefly, cells were incubated with NIV and DON, alone and in combination (10–100 μM) for 24 h, then washed in phosphate buffered saline (PBS) and re-suspended in 500 μL of a solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/mL PI. After incubation at 4 °C for 30 min in the dark, cell nuclei were analysed with Becton Dickinson FACScan flow cytometer using CellQuest program. Cellular debris were excluded from the analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. To elucidate apoptosis caspases involvement, NIV and DON (25 μM, concentration which gives a medium level of apoptosis) were added singly to J774A.1 in presence of a broad-spectrum caspase inhibitor, zVAD-fmk (50 μM). Data are expressed as the percentage of cells in the hypodiploid region.

2.4. Western blot analysis for Bax, ERK, pERK, caspase-3 and poly-ADP-ribose synthase (PARP)

J774A.1, murine monocyte/macrophage cell line (1.5×10^6/P60) was plated and allowed to adhere at 37 °C in a 5% CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with fresh medium and NIV and DON (25 μM) were added singly and cells incubated for additional 8 or 24 h. Total intracellular proteins were extracted from the cells by freeze/thawing in lysis buffer (50 mM Trizma–HCl). Protein content was estimated according to Biorad protein assay (BIO-RAD, Milan, Italy) and 50 μg protein/lane were loaded onto an acrylamide gel and separated by SDS-PAGE in denaturating conditions. The separated proteins were then transferred electrophoretically (100 mA per blot 45 min; Trans Blot Semi-Dry, BIO-RAD) to nitrocellulose paper (Immobilon-NC, Millipore, Bedford, USA) soaked in transfer buffer (25 mM Tris, 192 mM glycine, Sigma–Aldrich) and 20% methanol vol/vol (Carlo Erba, Milan, Italy). Non-specific binding was blocked by incubation of the blots in 5% no fat dry-milk powder (BIO-RAD) in TBS/0.1% Tween (25 mM Tris; 150 mM NaCl; 0.1% Tween vol/vol, Sigma–Aldrich) for 60 min. After washing, the blots were incubated overnight at 4 °C with the primary antibody anti-Bax, anti-ERK, anti-pERK, anti-caspase-3, anti-PARP or anti-tubulin, used as reference protein (all from Santa-Cruz Biotechnology, D.B.A. ITALIA s.r.l, Milan, Italy). After incubation with the primary antibodies and washing in TBS/0.1% Tween, the appropriate secondary antibody, either anti-mouse (diluted 1:1000) or anti-rabbit (diluted 1:5000), was added for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagents (ECL) and exposed to Hyperfilm (both from Amersham Biosciences, Milan, Italy). Films were then subjected to densitometric analysis using a Gel-Doc 2000 system (BIO-RAD).

2.5. Analysis of cell cycle

Cells, plated at 2×10^5/well, were exposed for 8 or 24 h to NIV and DON singly (50 μM). After incubation, macrophages were harvested and fixed in cold 70% ethanol at −20 °C. Cell cycle profiles were evaluated by DNA staining with PI (2.5 mg/mL) in phosphate-buffered saline (PBS) supplemented with 100 U/mL ribonucleases A, for 30 min at room temperature. Samples were analysed with a FACScan flow cytometer (Becton Dickinson, CA) using Mod FitLT program.

2.6. Data analysis

All values shown in table, figures and text are expressed as mean ± standard error of the mean of *n* observations, where *n* represents the number of observations. Data sets were examined by one-way analysis of variance and individual group means were then compared with Bonferroni or Student's unpaired *t*-test. A *P*-value less than 0.05 was considered significant.

3. Results

3.1. NIV and DON affect J774A.1 macrophage cell viability

After incubation of J774A.1 macrophages with graded concentrations of NIV and DON alone and in combination (10–100 μM) for 24, 48 and 72 h, cell viability was determined by MTT assay. The viability of control cells was designated as 100% and results have been expressed as the concentration of mycotoxin/s which induces the 50% of mortality in macrophages (IC_{50}). As shown in Table 1, a significant anti-proliferative effect was already observed at 24 h for NIV that is more powerful than DON in inducing cytotoxic effects on J774A.1 at all tested time ($P < 0.01$ at 24 h; $P < 0.05$ at 48 h and $P < 0.01$ at 72 h). Moreover, the contemporary presence of NIV and DON did not provoke any synergistic effect in inhibiting macrophage proliferation.

3.2. NIV- and DON-induced apoptosis in J774A.1 macrophages

In order to investigate the mechanism of anti-proliferative effect observed in NIV and DON treated macrophages a cytofluorimetric analysis was assessed incubating macrophages with mycotoxins

Table 1
Effect of NIV and DON, alone and in combination, on J774A.1 murine macrophage cell line viability. The viability of control cells was designated as 100%, and results were expressed as the concentration of NIV or DON able to induce the 50% of mortality in macrophages (IC_{50}). Results are expressed as mean ± s.e.m. from at least three-independent experiments.

Compound	IC_{50} [μM]		
	24 h	48 h	72 h
NIV	5.8 ± 0.9°°	10.5 ± 0.6°	11.2 ± 0.8°°
DON	23.3 ± 2.0	15.9 ± 0.3	16.8 ± 0.2
DON + NIV	15.0 ± 3.0	14.4 ± 1.5	14.0 ± 1.9

°° $P < 0.01$ NIV *vs*. DON at the corresponding experimental time.
° $P < 0.05$ NIV *vs*. DON at the corresponding experimental time.

(10–100 μM) for 24 h. Apoptosis was measured by cytofluorimetric analysis of PI staining of hypodiploid nuclei (Fig. 1). Our results indicate that both NIV and DON significantly stimulated apoptosis in J774A.1 macrophages in a concentration-dependent manner at all tested concentrations ($P < 0.001$ *vs*. control). Interestingly NIV pro-apoptotic effects at the concentrations of 25 and 50 μM resulted significantly higher than DON pro-apoptotic effect indicating a stronger pro-apoptotic activity for NIV ($P < 0.01$ *vs*. DON). No interaction was observed between NIV and DON pro-apoptotic effects (data not shown).

3.3. Induction of cell cycle arrest by NIV and DON

In order to determine if NIV and DON also affect macrophages cell cycle, J774A.1 cells were treated with 25 μM of each mycotoxin for 24 h. As depicted in Fig. 2, exposure for 24 h to both NIV and DON resulted in a significant increase of the G0/G1 cell population ($P < 0.001$ for DON *vs*. control and $P < 0.01$ for NIV *vs*. control) accompanied by a significant (DON *vs*. control: $P < 0.001$; NIV *vs*. control: $P < 0.01$) decrease in S phase cell population.

3.4. NIV- and DON-induced pERK expression in J774A.1 macrophages

In order to examine whether NIV and DON were able to influence ERK activation in J774A.1 macrophages, the effect of two trichothecenes was assessed. As shown in Fig. 3, both NIV and DON (25 μM) time-dependently induced ERK activation, as assessed by

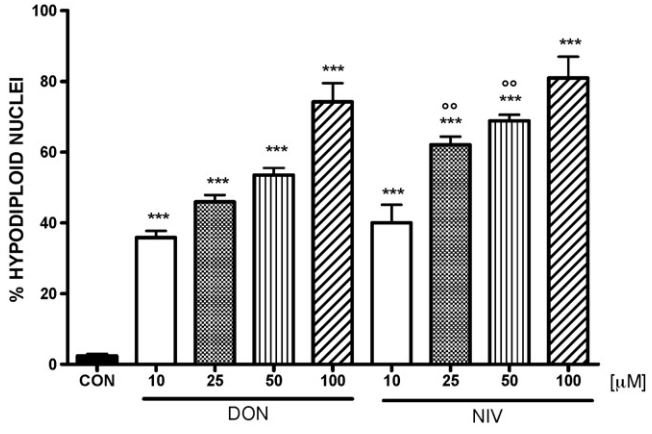

Fig. 1. Apoptosis detection by propidium iodide (PI) staining of hypodiploid nuclei. J774A.1 murine macrophages were incubated with NIV or DON (10–100 μM) for 24 h. Both NIV and DON exhibited a significant and concentration-related pro-apoptotic effect on J774A.1 macrophages (***$P < 0.001$ *vs*. control). NIV at the concentrations of 25 and 50 μM exhibits a stronger pro-apoptotic effect compared to the same concentrations of DON (°°$P < 0.01$). Data are expressed mean ± s.e.m. from at least three-independent experiments.

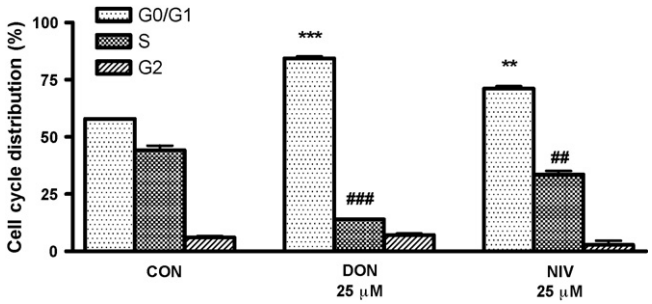

Fig. 2. Flow cytometric analysis of J774A.1 macrophage cell cycle phase distribution. Cells were treated with either NIV or DON (25 μM) for 24 h, incubated with PI and subjected to cell cycle analysis using a Becton Dickinson FACScan flow cytometer and ModFit software. Exposure to NIV or DON for 24 h resulted in a significant increase in the G0/G1 cell population (***$P < 0.001$ for DON *vs*. control and **$P < 0.01$ for NIV *vs*. control) accompanied by a significant decrease in S phase cell populations (###$P < 0.001$ for DON *vs*. control and ##$P < 0.01$ for NIV *vs*. control). Data are expressed mean ± s.e.m. from at least three-independent experiments.

pERK expression, in J774A.1 macrophages exposed to these trichothecenes for 15, 30 and 60 min. In NIV treated macrophages pERK resulted significantly expressed yet at 15 min indicating a faster activation of MAPK ERK by NIV ($P < 0.05$ NIV *vs*. control and *vs*. DON).

3.5. NIV and DON affects Bax expression

One of the main pro-apoptotic protein is Bax and we then examined whether NIV and DON are able to induce Bax activation. NIV and DON (25 μM) time-dependently induced Bax activation. As shown in Fig. 4, the effect of the two mycotoxins in activating Bax protein in J774A.1 macrophages is already evident and significantly enhanced after exposure of J774A.1 macrophages to 25 μM of both DON and NIV ($P < 0.001$ *vs*. control). Moreover after 8 h Bax expression resulted also significantly ($P < 0.001$) enhanced in cultured macrophages treated with NIV *vs*. those treated with DON.

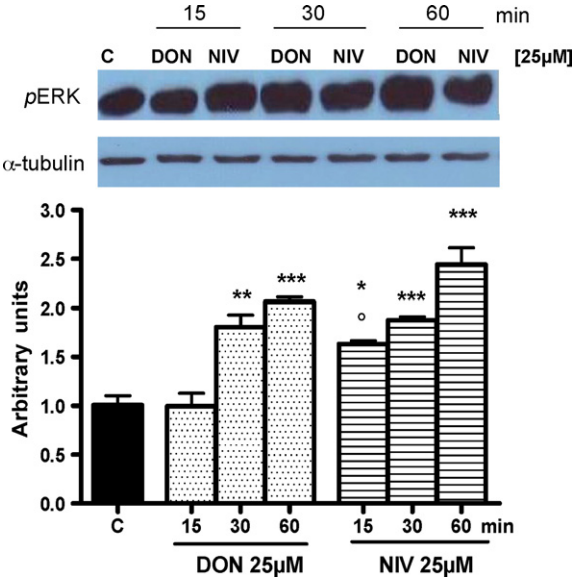

Fig. 3. Time dependent effect on pERK expression in J774A.1 macrophages treated for 15, 30 and 60 min with NIV or DON (25 μM). The activated form of ERK protein was detected by Western blot. Tubulin protein expression was used as a loading control. ***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$ *vs*. control; °$P < 0.05$ for NIV 15 min *vs*. DON at 15 min. The Western blot is representative of at least three-independent experiments.

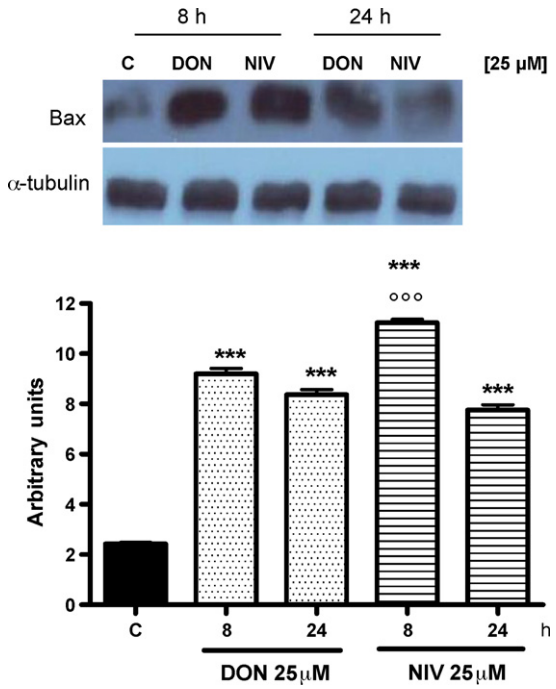

Fig. 4. Time dependent effect of NIV or DON on the expression of Bax pro-apoptotic protein in J774.A1 macrophages. Macrophages were treated with NIV and DON (25 μM) for 8 and 24 h. The activated form of Bax protein was detected by Western blot; $^{***}P < 0.001$ *vs.* control and $^{\circ\circ\circ}P < 0.001$ for NIV at 8 h *vs.* DON at 8 h. Tubulin protein expression was used as a loading control. The Western blot is representative of at least three-independent experiments.

3.6. Caspase activation by NIV and DON in cultured J774A.1 macrophages

In order to determine whether caspase activation could contribute to apoptosis induced in J774A.1 macrophages by NIV and DON, we exposed macrophages to trichothecenes in presence of zVAD-fmk, a broad-spectrum inhibitor of caspases. As shown in Fig. 5, zVAD-fmk (10 μM), a broad-spectrum caspase inhibitor, significantly inhibited the decrease in cell viability observed after exposure to 25 μM of NIV and DON, reducing percentage of

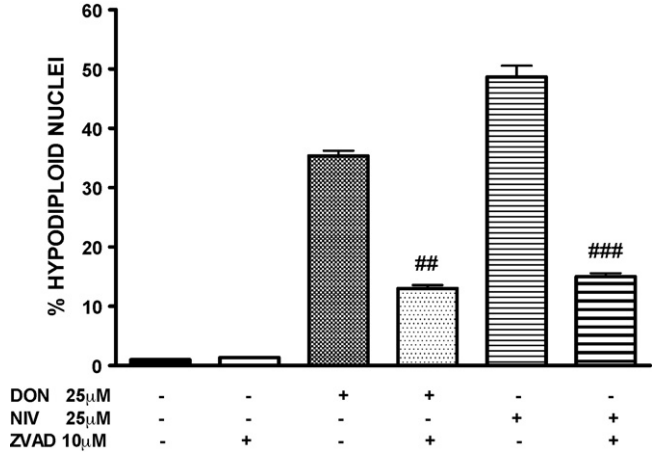

Fig. 5. Apoptosis detection by propidium iodide (PI) staining of hypodiploid nuclei with a broad-spectrum caspase inhibitor (zVAD-fmk). J774A.1 murine macrophages were treated with NIV or DON (25 μM) for 24 h in presence of zVAD-fmk (10 μM). The pro-apoptotic effect of NIV or DON was significantly reduced by the caspase inhibitor ($^{###}P < 0.001$ NIV + zVAD *vs.* NIV alone; $^{##}P < 0.05$ DON + zVAD *vs.* DON alone). Data are expressed mean ± s.e.m. from at least three-independent experiments.

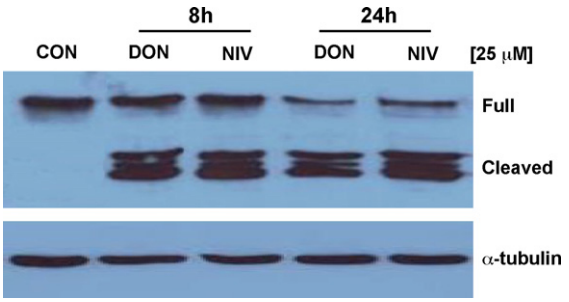

Fig. 6. Expression of the full and cleaved (activated) form of caspase-3. J774A.1 macrophages were treated with NIV or DON (25 μM) for 8 and 24 h. The cleaved form of caspase-3 was detected by Western blot. Tubulin protein expression was used as a loading control. The Western blot is representative of at least three-independent experiments.

hypodiploid nuclei from 48.3 ± 1.8 to 16.0 ± 1.1 ($P < 0.001$ zVAD-fmk + NIV *vs.* NIV alone) and from 38.0 ± 2.08 to 13.3 ± 0.9 ($P < 0.01$ zVAD-fmk + DON *vs.* DON alone).

3.7. Caspase-3 activation by NIV and DON in J774A.1 macrophages

Caspase-3 has been shown to lay downstream of the apoptotic signalling pathway. We also examined the effect of NIV and DON in inducing procaspase-3 degradation leading to caspase-3 activation. As shown in Fig. 6, NIV and DON (25 μM) time-dependently induced procaspase-3 degradation. The effect of these mycotoxins in activating caspase-3 in J774A.1 macrophages was already evident 8 h after incubation reaching the maximum effect 24 h after incubation with each mycotoxin.

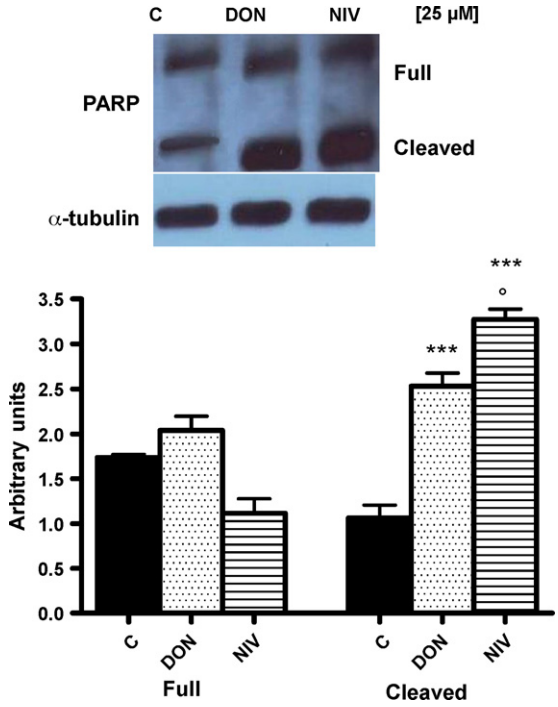

Fig. 7. Expression of the full and cleaved, activated, form of PARP. J774A.1 macrophages were treated with NIV and DON (25 μM) for 24 h. The cleaved form of PARP was detected by Western blot; $^{***}P < 0.001$ *vs.* control and $^{\circ}P < 0.05$ for NIV cleaved form *vs.* DON cleaved form. Tubulin protein expression was used as a loading control. The Western blot is representative of at least three-independent experiments.

3.8. NIV- and DON-induced PARP cleavage

To determine if the presence of NIV and DON could also affect DNA repair functions, we examined whether NIV and DON were able to induce PARP1 cleavage that leads to its activation. Our results showed that both NIV and DON are able to induce the activation of cleaved form of PARP1 after exposure of J774A.1 macrophages to NIV (25 μM) and DON (25 μM) for 24 h. As shown in Fig. 7, both mycotoxins are able to induce a significant ($P < 0.001$ *vs*. control) increase in activating PARP1 expression; in particular, the active form resulted further expressed in NIV treated macrophages ($P < 0.05$ *vs*. DON).

4. Discussion

The importance of studying trichothecenes toxicity is related to their food and feed contamination which induces severe consequences on both human and animal health. Human and animal cell lines have been used extensively to study the mechanism of the *in vitro* toxicity of *Fusarium* toxins. Exposure to trichothecenes such as NIV and DON resulted in decreased cell proliferation (Shifrin and Anderson, 1999) and it has been shown that macrophages are particularly responsive to these mycotoxins (Bae and Pestka, 2008; Pestka, 2008; Zhou et al., 2005; Sugita-Konishi and Pestka, 2001).

In this study we confirmed the cytotoxic effect of DON and, for the first time, we evaluated the effect of the mixture of NIV and DON on J774A.1 macrophage; moreover we demonstrated that NIV exhibits a stronger cytotoxic effect respect to DON on cultured J774A.1 macrophages and we studied the mechanisms underlying the toxicity of these trichothecenes. In particular, we showed that NIV resulted to possess anti-proliferative effects on murine macrophages and its cytotoxic effect could be partly ascribed to an acceleration of apoptotic pathway which involves (i) enhanced ERK activation, (ii) induction of pro-apoptotic protein Bax, (iii) induction of caspase-3 activation and (iv) activation of a DNA repairing enzyme, PARP. Moreover, treatment of J774A.1 macrophages with NIV and DON was also accompanied by an arrest of cell cycle in G0/G1 phase.

Results of the MTT assay revealed a significant decrease of viability of the J774A.1 macrophages treated with graded concentrations of NIV; this effect resulted stronger than decrease of cell viability in DON-treated cells at the same concentrations ($P < 0.01$) in according with another study on human erythroleukemia cell line, indicationg an impairment of macrophage mitochondrial cell metabolism after exposure to these mycotoxins (Minervini et al., 2004).

Food and feed commodities are often contaminated by more than one mycotoxin (Speijers and Speijers, 2004); among the several combinations that frequently occur, NIV and DON are often mentioned (Eskola et al., 2001). In terms of risk assessment, mycotoxins which show interactive effects are of more concern, in particular studying the molecular mechanisms of their toxicity to understand the real way by which mycotoxins can interact each other and exert immunomodulatory effects both in humans and animals (Luongo et al., 2008; Oswald et al., 2005). Finally, these studies can help to define or optimize the legal maximum limits of mycotoxins in food and feedstuffs. Nevertheless, there is relatively little information about interactions among co-occurring trichothecenes and the consequences for their toxicity on human and animal health. It is known that *Fusarium* toxins can exert additive and synergistic effects (Tajima et al., 2002) but mycotoxins can also act as antagonists (Koshinsky and Khachatourians, 1992). Luongo et al. (2006) showed that co-administration of α-zearalenol and fumonisin B1 was associated with an increased inhibition of Jurkat T cells proliferation in comparison to the effects of mycotoxins tested singly indicating a synergistic effect between these *Fusarium* toxins. The same authors reported a similar synergistic effect between α-zearalenol and fumonisin B1 on porcine blood immune cell proliferation while no interactive effects were observed between NIV and DON (Luongo et al., 2008). Theumer et al. (2003) showed different *in vitro* effects of a mixture of aflatoxin B1 and fumonisin B1 in comparison to the individual toxins. Another study on *Penicillium* mycotoxins showed that the majority of examined mixtures produced *in vitro* less-than-additive effects (Bernhoft et al., 2004). So, studying the interactions between different mycotoxins can be useful. The results of the current study showed that contemporary administration of NIV and DON on J774A.1 macrophages did not lead to a synergic cytotoxic effect indicating that their simultaneous presence did not enhance each own singular cytotoxicity in accordance with previous studies on different cell systems (Luongo et al., 2008).

High concentrations of trichothecenes promote apoptosis of many cell types such as macrophages (Yang et al., 2000). In order to investigate if the observed cytotoxic effect of NIV and DON was related to apoptosis induction we measured hypodiploid nuclei by cytofluorimetric analysis of PI staining. Interestingly our results indicated that NIV pro-apoptotic effect at the concentrations of 25 and 50 μM resulted significantly ($P < 0.01$) higher than DON-induced apoptosis at the same concentrations. These data are also confirmed by previous results indicating the capability of trichothecenes mycotoxins to increase apoptosis in macrophages (Zhou et al., 2005).

Analysis of cell cycle kinetics can give another insight into the mechanism of the effects of these mycotoxins on the macrophages proliferation. A significant reduction in the S-phase together with arrest of cells in the G0/G1-phase was observed for both NIV and DON *vs*. control cells indicating a cytostatic activity of these mycotoxins. Our data are tightly in accordance with previous studies in which cell cycle blocking in the same phase was observed in other cell lines incubated with DON (Tiemann et al., 2003). Moreover, it has been demonstrated that trichothecenes rapidly activate MAPK which contributes to the induction of apoptosis (Shifrin and Anderson, 1999). MAPK modulate physiological processes including cell growth, differentiation and apoptosis (Cobb, 1999); in particular ERK1/2 activation seems to be essential for inducing the competing apoptotic and survival pathways in macrophages exposed to trichothecenes (Yang et al., 2000). In our experimental system we observed ERK1/2 activation interfering with elements regulating cell cycle that could contribute to the observed cell cycle blocking and apoptosis. The intrinsic pathway of apoptosis involves translocation of Bax to the mitochondrial membrane and forming a pro-apoptotic complex. Our results showed that Bax expression was increased in macrophages treated with NIV and DON respect to control cells; Bax expression resulted augmented more at 8 h than at 24 h confirming the role of this protein in the early phase of apoptosis pathway. The pro-apoptotic complex, involving Bax, induces pores in the mitochondrial membrane leading to cytochrome *C* release and caspase-9 activation which activates caspase-3. Caspase-3 is an "executioner" that degrades a variety of cellular components producing apoptosis. In order to evaluate caspase-3 involvement in NIV- and DON-induced apoptosis, we exposed macrophages to trichothecenes in presence of zVAD-fmk, a broad-spectrum inhibitor of caspases. Incubation of J774A.1 with zVAD-fmk reverted NIV- and DON-induced apoptosis indicating an involvement of caspase in this process. Western blot analysis also showed an activation of caspase-3 in macrophages exposed to NIV and DON mainly at 8 h that could point out a good relation with Bax increase.

PARP activation is one of the conclusive aspects of apoptotic pathway (Szabò et al., 1997). There is evidence that the activation of PARP is an effector of apoptosis in presence of a strong stimulus of cellular damage (Andrabi et al., 2006) and in our study we observed

a significant increase of PARP activation in macrophages exposed to NIV and DON.

In conclusion, for the first time on J774A.1 macrophages, our results highlight the pro-apoptotic effect of NIV, a less studied trichothecene, whose toxic potential requires further studies because it is more toxic than DON and confirm the pro-apoptotic effect of DON, also showing another target of this trichothecene. Moreover, the results of the current study also showed no interactions between NIV and DON on J774A.1 macrophage viability.

Conflict of ixnterest

There is no conflict of interest.

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